ANALYSIS OF EXCRETORY/SECRETORY ANTIGENS OBTAINED FROM HELMINTH PARASITES

by

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Frontispiece: Cross section of mouse brain with migrating larva of *Toxocara canis* (x 1000).

Courtesy of Dr A. Stewart

This thesis is my own work, apart from those sections where appropriate acknowledgement is made.

T. Arulthilakan

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Abstract

Immunodiagnosis is important for the identification of human nematode larva migrans. It has been widely used for studying the epidemiology of human *Toxocara canis* larva migrans. However, cross reactions between immune sera and other parasites are a complication. Nematodes secrete a variety of antigenic proteins, as well as expressing antigens on their cuticles. ES antigens of *Angiostrongylus cantonensis, Toxocara canis, T. cati, T. pteropodis, Ascaris suum, Toxascaris leonina, Dirofilaria immitis and D. roemeri* were analysed by different methods. Most of these are potential human pathogens. The larvae of the first five parasites were successfully cultured *in vitro*, and their antigens were biosynthetically labelled with [^{35}S] methionine. Many of these adult parasites were surface labelled with ^{125}I. Antigens were dialysed, concentrated and their protein content was estimated. Their constituent peptides were separated by SDS-PAGE electrophoresis and located on the gels by Coomassie blue stain and autoradiography.

Each species secretes a number of different antigens into the culture media which are distinguishable by their molecular weights. The patterns of polypeptides released by the ascarids (*T. canis, T. cati, T. leonina, A. suum, T. pteropodis*) were similar to each other. There were less similarities between the ascarids and both the strongyle (*A. cantonensis*) and filaridae (*D. immitis* and *D. roemeri*). There appear to be stage and species specific peptides.

The cross reactions between parasites were investigated by immunoprecipitation and Western blot. Labelled antigens were incubated with appropriate sera from infected and uninfected hosts. The antigen antibody complexes were precipitated with *Staphylococcus aureus*, because protein A present on *S. aureus* binds IgG/antigen complexes thereby proving their antigenicity.

Another method, Western blot, was used to confirm antigenicity. In this method the antigens were transferred electrophoretically to a nitrocellulose paper (NCP). This NCP was incubated with antiserum to the parasite followed by enzyme linked second antibody which showed colour reactions on the antigenic bands in the presence of an enzyme linked substrate.

The degree of cross reaction and the nature of specificity was evaluated by Elisa tests.

CHAPTER 1

HELMINTH ZOONOSES

1.1 INTRODUCTION

Helminth parasites constitute an enormous medical and veterinary burden in many parts of the world. According to WHO estimates, Ascariasis is the most frequently recorded human parasitic disease (Schofield 1985). Over 200 million people are affected by filariasis in the world (Davis 1983). Helminth disease, in most cases can be readily diagnosed by morphological examination of eggs, larvae or adult parasites passed in the faeces or sputum or carried in the blood. There are, however, helminth infections in which identifiable parasite material is difficult to obtain. This is particularly true of zoonoses such as visceral larva migrans. It is in these infections that immunodiagnosis is potentially of great value. Parasitic nematodes are of great interest because most of their pathogenicity arises from their larval migration.

An attempt has been made in this thesis to characterize antigens from nematodes of zoonotic importance. The infective stages of the parasites were cultured *in vitro* with an aim of studying their species and stage specificity. They have been either surface or biosynthetically labelled and their excretory and secretory products purified and immunologically tested for their antigenicity.

The life cycle of nematodes can be divided into five stages, separated by four moults. There are no gross structural alterations between the newborn first stage larva and the adult (Chitwood and Chitwood 1950). Most nematodes are bisexual; the female fertilises her eggs with sperm deposited within her by the male. The female may then either release fertile ova or hatched larvae, depending on the species. Nematodes may be parasitic for all or part of their life cycle. Some, like filarial worms, pass through two hosts during their lives.

Table 1.1 Nematode parasites discussed in this thesis.

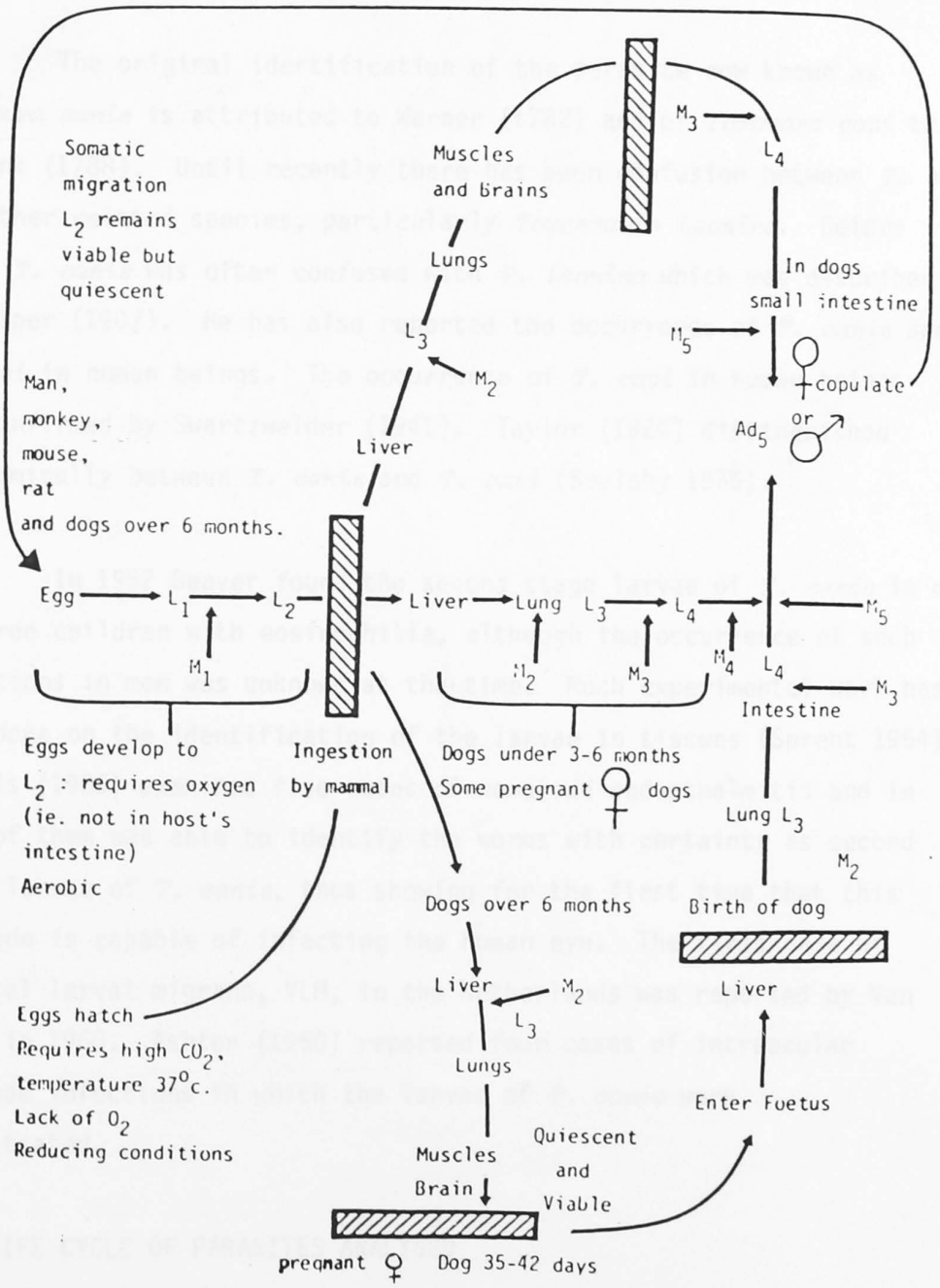
Species	Group	Host	Tissue location of adults	Infective for humans	Disease in man	Natural infection route
Toxocara canis	Ascaridae	Dog	Small intestine	Larvae, yes	VLM/blindness	Ingestion of eggs
T. cati	Ascaridae	Cat	Small intestine	Possibly larvae	Not recorded	Ingestion of eggs
T. pteropodis	Ascaridae	Fruit bat	Intestine	Possibly larvae	Suspected entritis and hepatitis	Ingestion of eggs
T. leonina	Ascaridae	Dog	Small intestine	Possibly larvae	Not recorded	———
Dirofilaria immitis	Filariae	Dog	Right ventricle of heart	Exceptional cases, larvae	Pulmonary nodules	Mosquito
D. roemeri	Filariae	Kangaroo	Connective tissue	No	Not recorded	Tabanid fly
Angiostrongylus cantonensis	Metastrongylidae	Rat	Pulmonary arteries	Yes, larvae	Encephalomylitis	With food

We can conveniently divide the various zoonotic helminth infections into four general categories (Beaver 1969):

1. Man is a normal and adequate, though unnatural, final host such as in the case of *Capillaria hepatica*, *Trichinella spiralis* and several species of *Trichostrongylus* and *Gongylonema*. These worms develop and behave in man essentially as they do in their natural host.
2. Man is a more or less normal, but inadequate final host; for example *Dirofilaria tenuis*, *D. immitis*, *Angiostrongylus cantonensis* and *Anisakis* sp. The behaviour and development of these worms up to a certain stage is the same in man as it is in natural final hosts. *D. immitis* and *D. tenuis* develop to sexual maturity, but only rarely reproduce (Pacheco and Schofield 1968). *A. cantonensis* reaches the fully differentiated adult stage in man but does not reach full maturity.
3. Man is a more or less normal but unnatural intermediate host, as with several larval tapeworms (eg. *Echinococcus granulosus*, *Taenia hydatigena*, *T. solium*) and ascarids such as *Amplicaecum* sp. In these examples the behaviour and development of the larvae is essentially the same in man as it is in the natural intermediate hosts.
4. In the fourth type of relationship; man is more or less a normal but unnatural paratenic host, for example *Toxocara canis*, *Gnathostoma* sp. and several other ascarids and spirurids. In these species, in which the natural life cycle involves a mammalian paratenic host, the larval behaviour in man can be regarded as reflecting the paratenic relationship.

In the last category, immunodiagnosis is useful because the larval parasites are difficult to find and identify. For immunodiagnosis

Fig. 1.1 Toxocara canis in dog with facultative two host cycle.



specific antigens are needed (Osimani 1957). This thesis analyses the antigens of *Toxocara canis*, *T. cati*, *T. pteropodis*, *Toxascaris leonina*, *Ascaris suum*, *Angiostrongylus cantonensis*, *Dirofilaria immitis* and *D. roemeri*. Most of the above parasites cause zoonoses in human beings.

The original identification of the parasite now known as *Toxocara canis* is attributed to Werner (1782) and of *Toxocara cati* to Schrank (1788). Until recently there has been confusion between *T. canis* and other related species, particularly *Toxascaris leonina*. Before 1907, *T. canis* was often confused with *T. leonina* which was described by Leiper (1907). He has also reported the occurrence of *T. canis* and *T. cati* in human beings. The occurrence of *T. cati* in human beings was confirmed by Swartzwelder (1941). Taylor (1924) distinguished taxonomically between *T. canis* and *T. cati* (Soulsby 1976).

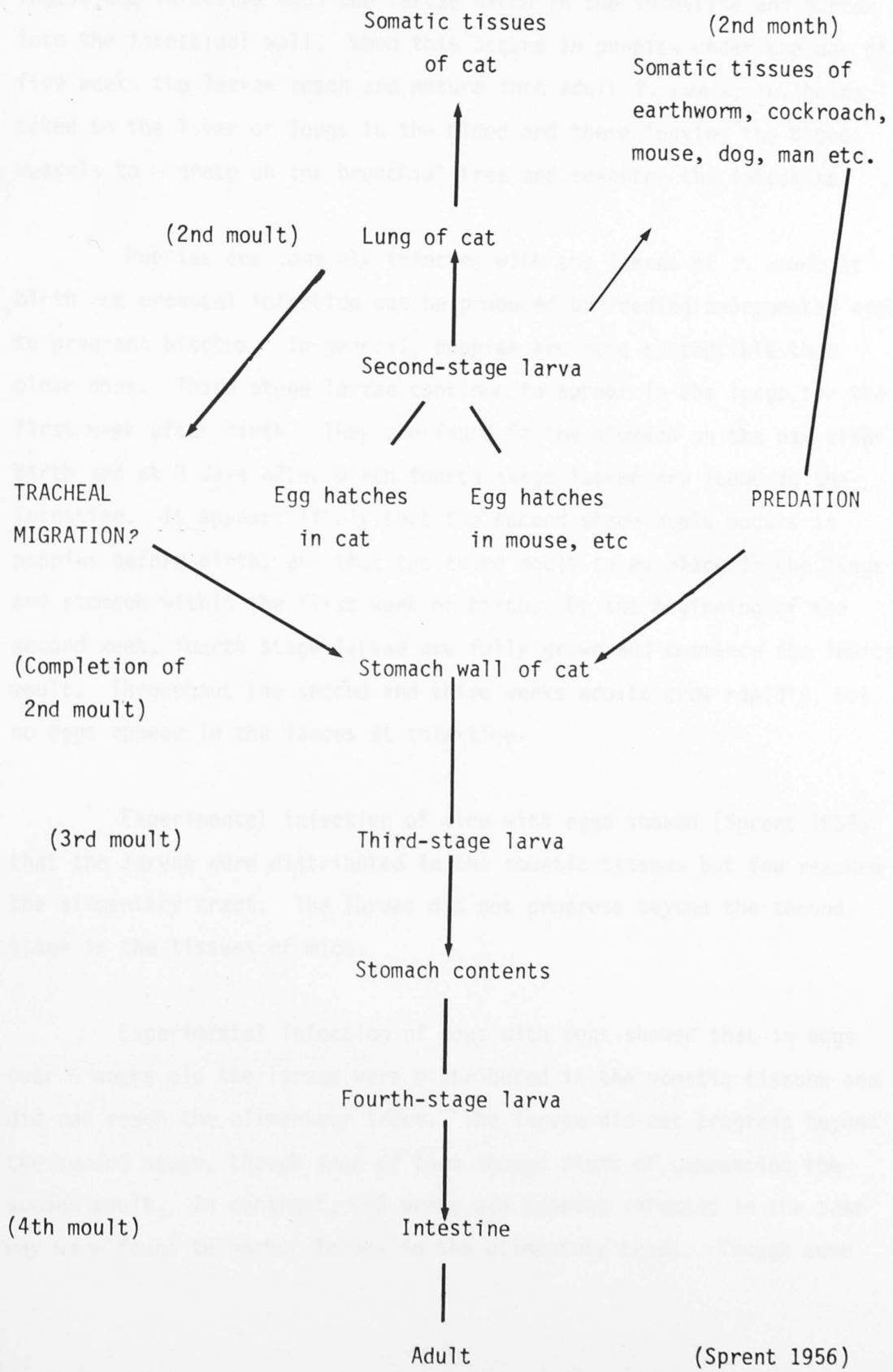
In 1952 Beaver found the second stage larvae of *T. canis* in one of three children with eosinophilia, although the occurrence of such infections in man was unknown at the time. Much experimental work has been done on the identification of the larvae in tissues (Sprent 1954). Nichols (1956) examined five cases of nematode endopthalmitis and in four of them was able to identify the worms with certainty as second stage larvae of *T. canis*, thus showing for the first time that this nematode is capable of infecting the human eye. The first case of visceral larval migrans, VLM, in the Netherlands was reported by Van Thiel in 1960. Ashton (1960) reported four cases of intraocular nematode infections in which the larvae of *T. canis* were demonstrated.

1.2 LIFE CYCLE OF PARASITES ANALYSED

1.2.1 *Toxocara canis*

The full development of *T. canis* and *T. cati* were first described by Sprent in 1958. The adult toxocaral worms are found in the

Fig. 1.2 A schematic representation of the life history of *T. cati*



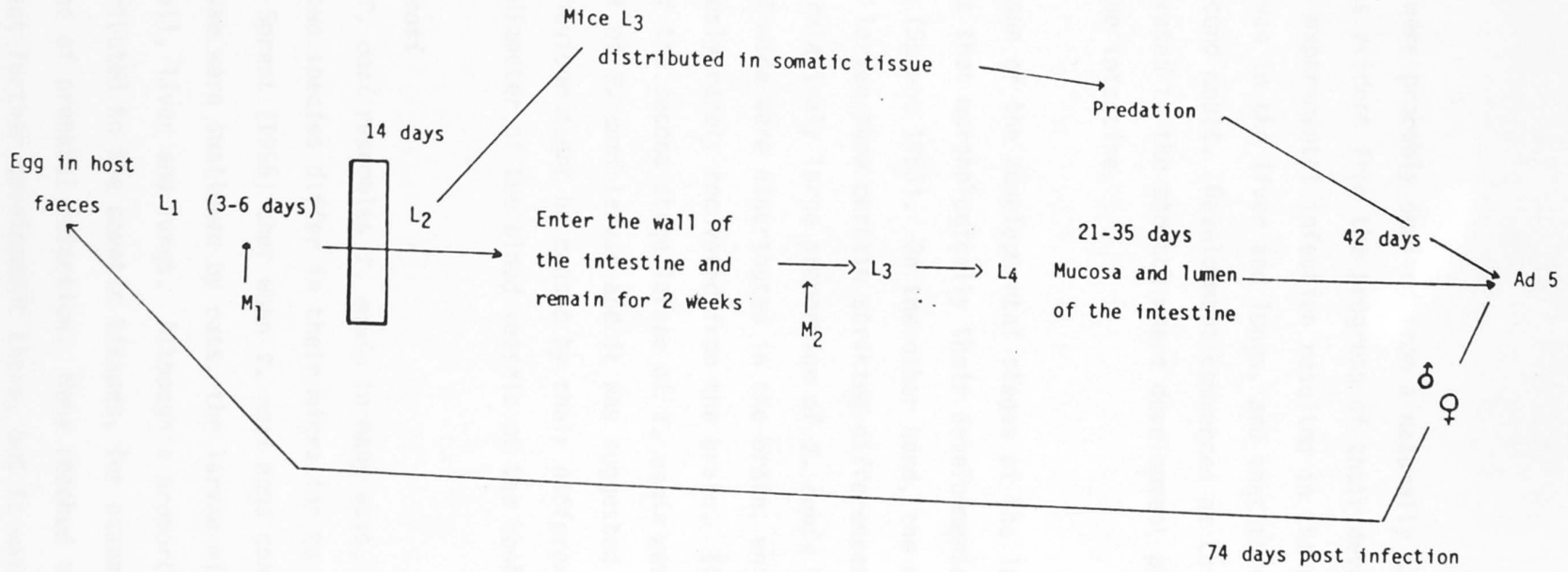
intestines of dogs and cats respectively. When the natural host or man ingest the infective eggs the larvae hatch in the intestine and burrow into the intestinal wall. When this occurs in puppies under the age of five weeks the larvae reach and mature into adult *T. canis*, ie. being taken to the liver or lungs in the blood and there leaving the blood vessels to migrate up the bronchial tree and re-enter the intestine.

Puppies are commonly infected with the larvae of *T. canis* at birth and prenatal infection can be produced by feeding embryonated eggs to pregnant bitches. In general, puppies are more susceptible than older dogs. Third stage larvae continue to appear in the lungs for the first week after birth. They are found in the stomach on the day after birth and at 3 days after birth fourth stage larvae are found in the intestine. It appears likely that the second stage moult occurs in puppies before birth, and that the third moult takes place in the lungs and stomach within the first week of birth. By the beginning of the second week, fourth stage larvae are fully grown and commence the fourth moult. Throughout the second and third weeks adults grow rapidly, but no eggs appear in the faeces at this time.

Experimental infection of mice with eggs showed (Sprent 1958) that the larvae were distributed in the somatic tissues but few reached the alimentary tract. The larvae did not progress beyond the second stage in the tissues of mice.

Experimental infection of dogs with eggs showed that in dogs over 5 weeks old the larvae were distributed in the somatic tissues and did not reach the alimentary tract. The larvae did not progress beyond the second stage, though some of them showed signs of commencing the second moult. In contrast, 1-3 weeks old puppies infected in the same way were found to harbor larvae in the alimentary tract. Though some

Fig. 1.3 A schematic representation of the life history of *T. leonina*



of these larvae were probably derived from a naturally acquired prenatal infection, it was evident from the progress of their development in the puppies that the experimental infection resulted in the presence of second stage larvae in the liver and lungs, and that these larvae underwent the second moult. Development commenced as third stage larvae in the lungs migrated to the stomach where development proceeded to the adult stage in the intestine.

Comparison of the developmental stages of the larvae of *T. canis* and *T. cati* shows that morphologically their developmental stages are almost identical (Sprent 1956). On the other hand, the migratory behaviour of the larvae show certain striking differences. Sprent (1956) reported that a relatively large proportion of *T. canis* larvae migrating in the tissues of mice were distributed in the brain, while the larvae of *T. cati* were only rarely recovered from the brain. It was found that the width of the second stage larvae of *T. canis* was slightly greater than that of *T. cati* larvae and it was suggested that their difference in behaviour might be caused by their difference in width, in relation to the diameter of the blood vessels of the host (Sprent 1955).

1.2.2 *Toxocara cati*

Though *T. cati* resembles *T. canis* in many ways, the larvae of these two species differ in their migration to foetal tissues. It was shown by Sprent (1956) that when *T. cati* eggs containing second stage larvae were swallowed by cats, the larvae migrated through the intestinal wall, liver and lungs. Although a proportion of these larvae were distributed to the somatic tissues, for example muscles, no evidence was found of prenatal infection. Many reached the alimentary tract and underwent further development there, but it was not established whether they reached this site by tracheal migration. The behaviour of *T. canis* larvae, in the same circumstances, differed in that the larvae

were evidently unable to reach the alimentary tract of dogs, except in very young animals (birth to 3 weeks), but migrated in relatively large numbers to the somatic tissues.

Cats, dogs, lambs, mice, chickens, cockroaches and earth worms can be experimentally infected with *T. cati* eggs. In egg-infected cats the larvae were found in the liver, lungs, muscles, and tracheal washings as well as in the digestive tract, indicating that they migrate through the tissues. In mice the larvae were found in liver, lungs and muscles with very few or any in the brain, in contrast to *T. canis*, and they did not reach the alimentary tract. In cats fed both with eggs and with infected mice the third stage larvae grew in the stomach wall and moulted for the third time. This occurred at about 10 days in cats fed with infected mice and at about 19 days in egg-infected cats. Sexual differentiation becomes evident during this stage. Eggs were first observed in the faeces at 56 days after infection by eggs.

1.2.3 *Toxocara pteropodis*

Another member of the Ascaridea, *Toxocara pteropodis*, was described by Baylis (1936) from Vanuatu. It was found in the intestines of suckling fruit bats, *Pteropus geddiei*. One adult female worm releases 20,000-30,000 eggs per 24 hours, intermittently rather than continuously (Prociv 1983). Eggs are at the one cell stage when released in the faeces and cell division commences within several hours of being passed. Motile larvae are present on the fifth day and on the tenth day virtually all eggs are fully embyronated. *T. pteropodis* undergoes transmammary transmission, which occurs within 2 weeks of the birth of the young bat. All the larvae in the mother bats liver migrate to the mammary glands at about the time of parturition. These larvae then pass in the milk to the neonate in the first few days after birth, and perhaps over a period as long as 2 weeks. Larvae were never found in

the lungs of young bats (Prociv 1983), indicating that tracheal migration does not occur. Developing worms mature and commence producing eggs after about 2 months.

An outbreak of acute hepatitis and gastroenteritis was reported amongst children on Palm Island in November 1979 (Byth 1980). It was suggested by Moorhouse (1982) that it may have been caused by epidemic visceral larva migrans resulting from the ingestion of *T. pteropodis* eggs. Suckling fruit bats defaecate over fruit, while attached to their mothers, when the mothers are feeding on fruit, and eggs have been found in their faeces on the fruits of mangoes.

1.2.4 *Toxascaris leonina*

T. leonina Linotow (1902) occurs in the small intestine of the dog, cat, fox and wild Canidae and Felidae in most parts of the world. The infective stage is the egg containing a second stage larva. This, under optimum conditions outside the host, is reached in 3-6 days. Following ingestion and hatching, second stage larvae enter the wall of the intestine and remain in this site and this stage for about 2 weeks. Moulting to the third stage larvae commences about 11 days after infection and is followed fairly quickly by a moult to the fourth larval stage. Fourth stage larvae are present in numbers 3-5 weeks after infection. At this stage they are in the mucosa and the lumen of the intestine. Fifth stage adults are produced about 6 weeks after infection and eggs are produced from 74 days onwards. No larval migration occurs, unlike *T. canis*. Larvae of *T. leonina* may occur in mice. In this animal, third stage larvae are distributed in many tissues and if an infected mouse is eaten by a dog or cat, the larvae are ingested from the mouse tissues and develop to maturity in the wall and lumen of the intestine of the final host. Whereas larvae in the dog and cat are restricted to the intestine, in the mouse they migrate out of this site being distributed all over the body. Sprent (1959) considers this

indicative that intermediate hosts are fully utilised in the life history of the parasite. Leipier (1907) has reported this infection in man.

1.2.5 *Ascaris suum*

For many years *Ascaris suum* (Goeze, 1782), naturally occurring in pigs was considered synonymous with the human parasite *A. lumbricoides* Linnaeus, 1758, but there is now good evidence that the two are distinct species. Serological differences have been reported in the carbohydrate fractions of the two Campbell (1937). Sprent (1952) has described morphological differences in the denticulation of the lips of the two similar species.

The eggs are passed in the faeces of the host and develop to the infective stage in 10 days or longer, depending on the temperature. The eggs are very resistant to adverse conditions. During development each larva moults once within its egg shell to become a second stage larva. This is the infective stage. Infection takes place through the ingestion of eggs with food or water or from soiled skin of the mother while suckling. The ingested eggs hatch in the intestine and the larvae burrow into the walls of the gut. They may pass through into the peritoneal cavity and thence to the liver but the majority reach this organ by the hepatoportal blood stream. They may arrive in the liver 24 hrs after the eggs have been ingested, or even earlier. From the liver they are carried by the blood through the heart to the lungs where most are arrested in the capillaries, although some may pass through into the arterial circulation and reach other organs such as the spleen and kidney. The majority of the larvae moult to the third stage between 4th and 5th day after infection. At this time many larvae are still in the liver though a good proportion have migrated to the lungs.

The act of moulting initiates a period of marked growth and development which is also associated with the migration of larvae to the lungs. Larvae break out of the alveolar capillary into the alveolus and pass through the alveolar duct to the small bronchioles and then gradually ascend the bronchial tract. Larvae then migrate from the trachea to the pharynx, whereupon they are swallowed. The stage three larvae arrive in the intestine 7-8 days after infection.

Douvres (1967) states that the moult to the fourth larval stage occurs about the 10th day, in the intestine, but on the contrary Roberts (1934) states that this moult occurs in the respiratory system and only the fourth larval stage are able to survive the acidic environment of the stomach (Soulsby 1976). Large numbers of fourth stage larvae are present in the small intestine between the 14th and 21st day after infection. The moult to the fifth stage, or young adult, occurs 21-29 days after infection. Maturity occurs after 50-55 days and eggs appear in the faeces at 60-62 days. Soulsby (1965) has given a detailed account of the morphology of the different stages. The eggs of *A. suum* will hatch and the larvae migrate in many animal species, including man. Eggs are not usually produced although accidental human infection in laboratory workers using *A. suum* has been noticed (Soulsby 1976). There is no evidence of prenatal infection by *A. suum*.

1.2.6 *Dirofilaria immitis* (Leidy 1856)

As a member of the Filarioidea, its life cycle involving an intermediate host vector is quite different from the previously discussed Ascarids. It is included here because of possible cross reaction with ascarids (Pachaco 1966; Tulloch 1970).

This is the common heart worm of dogs, cats, foxes and wolves. The adult worm lives in the dog for about 5 years in the right ventricle

and the pulmonary arteries. They cause pulmonary endoarteritis, while dead worms may cause embolism and granulomatous reactions (Adcock 1961). The adult worm produces microfilariae within 4-6 months after infection by the mosquito. The microfilaria shed the shell membrane and are found in the peripheral circulation during which time they are transmitted to the mosquito. Taylor (1960) has described the development of microfilaria in the mosquito. After development and moulting in the mosquito, the infective third stage larvae enter the host when the mosquito bites the dog. The microfilaria may be more pathogenic than was initially appreciated (Otto 1969). The microfilariae are ingested by many species of mosquitoes and develop to third stage larvae within 17 days (Knight 1977). The third stage larvae live only a few days in the proboscis of the mosquito and must be injected into the dog within that time to complete that cycle. The role of the migrating larvae in the dog is still unkown, but they have been found in abnormal sites such as the eye and it is possible that they may cause inflammatory and antibody antigen responses. The third stage larvae moult to the fourth stage about 9-12 days after infection and to the fifth or adult stage 60-70 days after infection (Kume 1967).

Some human cases have been reported. Billups, Scheriken and Beaver (1980) reported subcutaneous dirofilariasis in Nebraska, where, in addition to pulmonary nodules, *D. immitis* caused symptomatic subcutaneous nodules. Sato and co-workers (1985) reported human pulmonary dirofilariasis in Japan. Dirofilariasis by other species is reported, eg. *D. megalhaesi* is reported from the left ventricle of a Brazilian child and *D.louisianensis* from the inferior vena cava of an elderly Negress in New Orleans (Cheng 1973). Subcutaneous dirofilariasis, probably caused by *D. repens*, was reported in Sri Lanka (Attygalle and Dissanaike 1970; Ratnavale and Dissanaike 1964; Wijetileka, Attygalle and Dissanaike 1962). Its widespread distribution in the human body is described by Dissanaike, Lykov, Sri Skandarajah Sivayoham and Perera

(1972). The importance of this zoonotic infection has recently been high-lighted and over a hundred cases of subcutaneous dirofilariasis have been reported from man in many parts of the world (Dissanaike 1979).

1.2.7 *Dirofilaria roemeri*

For comparative purposes an attempt to analyse the antigens from *D. roemeri* infecting marsupials, is reported in this thesis. *D. roemeri* has a similar life cycle to that of *D. immitis*, except that their site of predilection is the kneecap, and the vector is a Tabanid fly (Spratt 1975).

1.2.8 *Angiostrongylus cantonensis*

A. cantonensis was first observed in the lungs of rats in Canton, China by Chen (1933). Sexually mature males and females of *A. cantonensis* are normally found in the two main branches of the pulmonary artery (Fig. 1.4), and occasionally in the right ventricle of various species of rats (Fig. 1.5). Free-living infective first, second and third stage larvae are found in a molluscan intermediate host (Fig. 1.6). The remainder of the third and fourth stage larvae and the adults (fifth stage) are found in the mammalian host.

When the third stage larva is ingested by the mammalian host it migrates to the central nervous system where it grows slightly, and on the fifth day post infection it moults for the third time to reach the fourth stage. On the 10th day post infection some females complete their fourth moult while males complete this moult on the eleventh day. The young adult worms, which are found on the surface of the rat brain (sub arachnioidal space) (Fig. 1.7), undergo further growth until the 26th day post infection, when they start penetrating the engorged venules and travel to the lungs to reach sexual maturity.

Fig. 1.4 Adult *A. cantonensis* found in the main branch of the pulmonary artery.

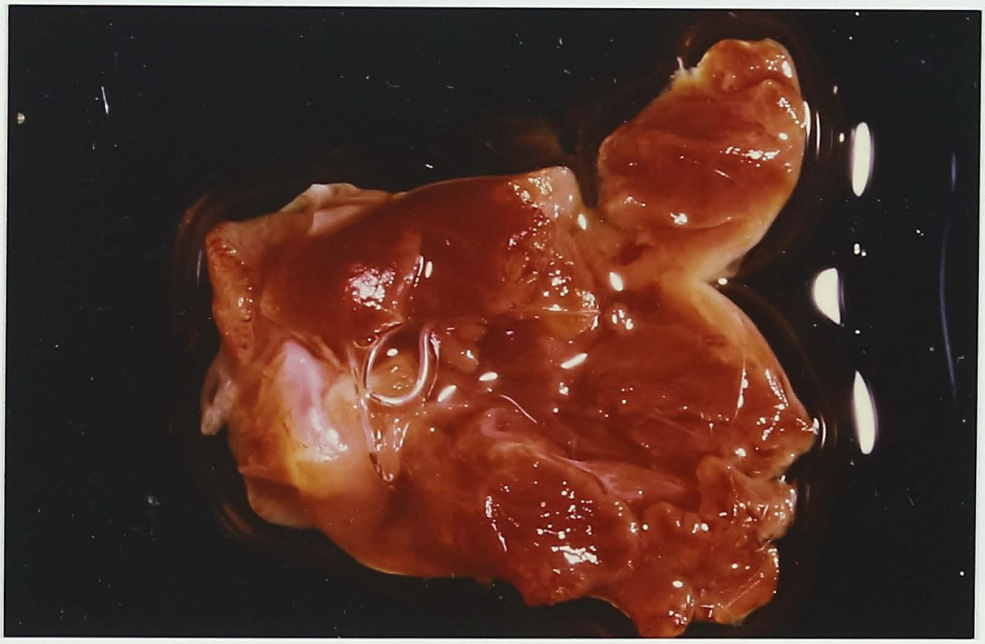


Fig. 1.5 Adult *A. cantonensis* found in the right ventricle of the rat heart.

Fig. 1.6 *In vitro* cultured third stage larvae which was obtained from snails (x 800).

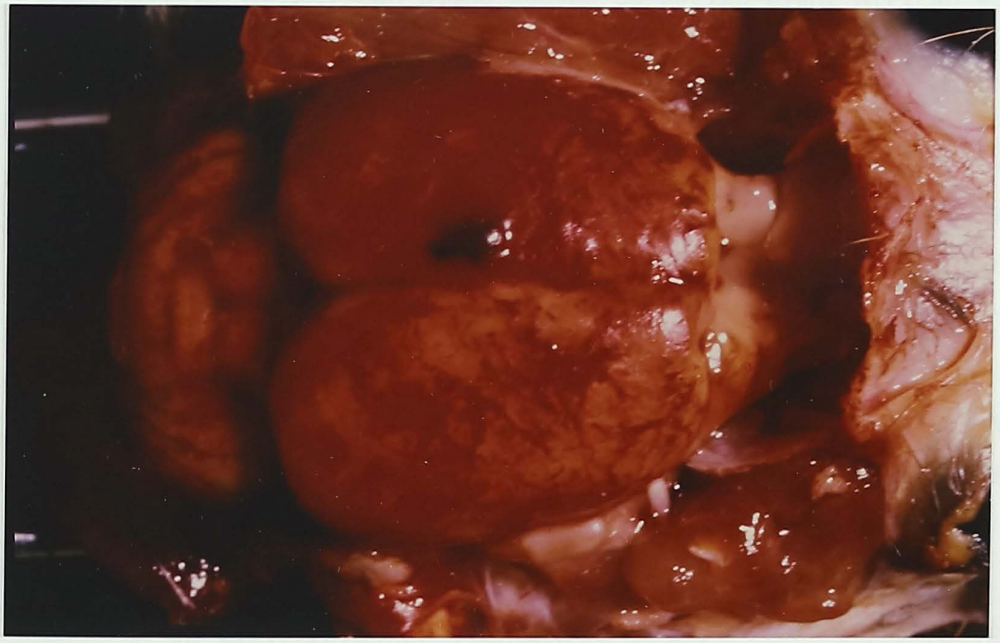


Fig. 1.7 The young adult worms of *A. cantonensis* found on the sub-arachinoidal space of the rat brain. Haemorrhage due to penetration of the engorged venules by the migrating parasites.

Human beings are exposed to the infection by ingestion of the 3rd stage larvae (Fig. 1.6) in either a snail or a contaminated freshwater prawn, *Macrobrachium* sp., native foods that include prawn juice or fresh vegetables and fruits on which there are infected slugs. Alicata in 1961 incriminated the parasite as a possible cause of eosinophilic meningioencephalitis. Alicata (1970) reported a case of the disease in Hawaii resulting from the ingestion of raw slugs. Since then it has frequently been observed in South East Asia and the Pacific Islands (Rosen *et al.* 1962).

In 1981 the first case was reported in Cuba by Aguiar, Morera and Pascual-Enzenaver and Yamaoka (1982) demonstrated an eosinophilic meningitis and hydrocephalus in an infant in the USA. The same year Kliks, Kroenk and Hardman (1982) reported a case of radiculomyelo-encephalitis in American Samoa caused by eating giant African snails (*Achatina fulica*) infected with *A. cantonensis*.

Alicata (1966) reported the infection in 64% of bandicoots in Sri Lanka and nearly 30% of *Rattus norvegicus*. The Giant African snail *Achatina fulica*, which is quite a common garden pest in Sri Lanka, is probably an intermediate host. Although the habit of eating anything raw or under cooked, least of all snails and slugs, is rare in Sri Lanka, it is possible for infection to occur through accidental ingestion with vegetables of the free infective larvae from damaged snails or slugs.

In addition to *A. cantonensis* in north Queensland (Mackerras and Sandars 1955), a related species, *A. mackerrasae* has recently been reported from rats in Brisbane, (Bhaibulayo 1968). Furthermore, *A. vasorum* is also known to be present among dogs in Australia (Alicata 1970).

1.3 NEMATODE ANTIGENS

Parasite antigens may be classified in a number of ways (Anders, Howard and Mitchell 1982). Nematode antigens were originally defined by parasitologists and so the criteria used were the stage of the life cycle and the source. For the convenience of classification, most animals can be divided into three simple and operational compartments, namely (a) the surface, (b) the excretory-secretory (E/S) and (c) the residual somatic compartments. Until recently little attention was paid to the surface and the other two compartments were used without any serious purification procedures. With the use of metabolic or enzymic radiochemical labelling procedures it is possible to identify the various components of these compartments. Once the surface, for example, is radiolabelled, it is possible to monitor the distribution of its components through various purification procedures, such as electrophoresis, chromatography and immunopurification. Some of these characterisation methods are dealt with in subsequent chapters of this thesis.

For many years the cuticle of nematodes was thought to be a relatively inert acellular exoskeleton surrounding layers of muscle (Lumsden 1975). Its composition has not been fully elucidated (Lumsden 1975; Bird 1980; Lee 1972) but it is known to consist of three or more layers of varying thickness. The external surface is delineated by a triple layered structure which bears resemblance to a mammalian cell membrane. However, freeze fracture studies of the surface of two very different parasitic nematodes, namely the microfilaria of *Onchocerca volvulus* (Martinez Palomo 1978) and the adults of *Trichinella spiralis* (Lee, Wright and Shivers 1984), have clearly demonstrated that the outer surface of nematodes is not delimited by a membrane like the plasmalemma which surrounds eukaryotic cells. Despite its very different structure from the mammalian cell surface, the cuticle of nematodes is, in contrast to the earlier hypotheses, a very active

structure. The nematode cuticle is moulted four times during development with changes on each moult. The new cuticle is synthesised beneath the old cuticle by self-assemblage of molecules secreted by the hypodermis with the exocuticle forming first. Enzymes and haemoglobin have been located in it and the adult stages of *Brugia pahangi* have been shown to take up nutrients via their surfaces (Chen and Howells 1979; Howells and Chen 1981). More direct evidence of the dynamic nature of this structure was first obtained from analyses of the radiolabelled surface proteins of *Trichinella spiralis* (Maizels, Phillip and Ogilvie 1982).

An understanding of why different nematode species have evolved different patterns of expression of surface antigens will require the functional characterisation of these molecules. It is clear, nonetheless, that the cuticle is capable of surprisingly dynamic changes at the molecular level, including the ability of secreting protein molecules synthesised after the cuticle itself has been fully completed and has replaced that of the previous developmental stage (Maizels, Meghi and Ogilvie 1983; Jungery, Clark and Parkhouse 1983; Philipp, Taylor, Parkhouse and Ogilvie 1981). There is good evidence that the proteins labelled were on the surface of the worms. The number of peptides separated by gel-electrophoresis revealed for each stage was highly restricted, with no sign of widespread labelling throughout the worm. In addition, the surface location of the radioactivity was directly demonstrated by autoradiography of sections of the labelled worms (Parkhouse *et al.* 1981). Finally, Maizels *et al.* (1983) have used the same technique to label the surface proteins of *Nippostronglyus brasiliensis* but failed to find any labelling of nematode-derived haemoglobin, a protein known to be located in the cuticle close to the surface.

1.3.1 Nematode Antigens for Diagnosis

The prime aim of the drive to characterise nematode antigens is for their application in diagnosis of nematode infections of man and animals. Studies have concentrated on developing some form of serological test which provides the specificity and sensitivity required. Serological tests have involved complement fixation, haemagluttination and fluorescent antibody tests. More recently, enzyme-linked immunosorbant assay (Elisa) techniques have been applied. The Elisa has been demonstrated to be up to 100 times more sensitive than other serological techniques in assaying for trichinellosis in a variety of hosts (Ruitenberg *et al.* 1975; Van Knapen *et al.* 1980). However, in the case of serological assays for diagnosis of filarial infections, the improved sensitivity of the tests has been overshadowed by other problems. As the reviews by Kagan (1963, 1982) and Ambroise-Thomas (1980) have high lighted, the primary problem encountered is cross reactivity in the test. This is due to the use of crude antigenic extracts and sera from patients with incomplete medical records. Hopefully, diagnostic studies using defined parasitic antigen-antibody reactions will overcome these problems.

Surface or metabolic labelling provides us with the means to select one or more parasite antigens from specific compartments of the worm. Earlier work demonstrated that there are a restricted number of stage- and species-specific antigens on the surface or in the secretions of *T. spiralis* (Philipp *et al.* 1981; Parkhouse and Clark 1983), *N. brasiliensis* (Maizels *et al.* 1983) and *T. canis* (Maziels *et al. 1984). In the case of T. spiralis*, the results obtained from the analysis of radiolabelled proteins helps to explain the remarkable specificity of the Elisa assay for trichinellosis when using the crude saline soluble fraction from homogenised muscle larvae (Ruitenberg *et al.* 1975). As mentioned above, the surface labelled proteins of muscle larvae of *T. spiralis* are both stage and species

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specific (Phillip*et al.* 1980; Parkhouse *et al.* 1981). Furthermore, studies of metabolically labelled proteins demonstrated that secreted proteins displayed similar properties (Parkhouse and Clark 1983). Finally, when muscle larvae were metabolically labelled with [^{35}S]-methionine, none of the labelled solubilised somatic proteins were precipitated by sera from infected mice. This suggests that most of the antigenic proteins analysed so far are stage and species specific, accounting for the specificity of this Elisa test.

In contrast to the nematodes studied earlier, filarial worms present a more complicated picture. The surfaces of *Litomosoides carinii* do not have totally exclusive stage-specific determinants (Philipp *et al.* 1984). Similarly, the major surface labelled proteins of brugian filariae are cross reactive, not only between stages, but also between species (Maizels *et al.* 1983). In fact, the cross reactivity between stages is so strong that mice primed with microfilariae and then challenged with infective larvae produce an antibody response to surface determinants of the third stage larvae (Maizels *et al.* 1983; Jeska 1967, 1969). The major surface protein of *Onchocerca gibsoni* displayed even greater cross reactivity when tested against a panel of sera taken from patients with a variety of filarial and trematodal infections (Forsyth *et al.* 1981).

However discouraging these results may seem, the possibility should not be overlooked that one or more of the minor proteins in a specific compartment may be totally specific for either the species or the stage of the parasite. The ability to label protein from each compartment selectively enables us to look systematically for potentially immunodiagnostic protein in each compartment. One may speculate that the surface and secretion of the parasite are more likely to contain the species and stage specific antigens which may be needed for parasites to survive in the different environments encountered

during its life cycle. In contrast, the basic, functional or metabolic molecules found in the somatic fraction will be more conservative, not only through the life cycle, but also across species. It is important to note that radiolabelling procedures do not necessarily tag all of the antigenic components. Glycolipids and carbohydrate moieties are not labelled with radioactive amino acids, nor perhaps are all the proteins. For example, Kaushal *et al.* (1982) found that iodination of the E/S components of microfilariae of *Brugia malayi* produced an unexpectedly specific "diagnostic" antigen. Here, although the E/S fraction was complex as judged by Coomassie blue staining of proteins separated by SDS PAGE, similar analysis of the iodinated protein revealed that only a restricted number of the smaller molecular weight component were labelled and these were presumably species specific.

By the application of these techniques Phillip *et al.* (1984) identified a potentially useful protein for diagnosis on the surface of adult stages of *Onchocerca volvulus*. It has a MW of 20,000 and promising results were obtained when it was used in a serological survey of people living in an area endemic for onchocerciasis in Southern Mexico. The 20-KD-labelled component from the surface was separated from the other labelled surface protein by gel filtration and then used in an immuno-precipitation test with sera from people living in villages either endemic or non-endemic for the disease. The specificity of the test was 98% and the sensitivity 92%. Of interest was the detection of anti-bodies in sera from patients living in the endemic area, but without signs of clinical disease. These probably represent examples of people with prepatent infections, or who have been exposed to the worms without developing disease.

Specific antigens are required for an assay which is of high specificity. They need not, however, be derived from the parasite detected in the assay. There are examples of specific cross-reactions

between nematodes which have been exploited for diagnosis. For example, Dissanayake and Ismail (1980), reported that fractions of a detergent-soluble extract of adult worms of *Setaria digitata* (a nematode infection of cattle) separated on DERE-Sephadex columns were cross-reactive with *Wuchereria bancrofti*. Some of these components cross reacted with major antigens of the microfilariae since one of the *S. digitata* extracts blocked the binding of immune sera to microfilaria of *W. bancrofti*. Another fraction designated SD 2.4 was used as an antigen in an assay for bancroftian filariasis. The antigen cross reacted reacted with a component of adults of *W. bancrofti* (Dissanayake *et al.* 1980). Subsequently, Dissanayake and Ismail (1982) isolated immune complexes from the sera of microfilaraemic patients and found that these complexes contained the component which cross reacted with SD 2.4. Another potentially useful cross reactivity identified was a fraction of adults of *Litomosoides carinii* detected by sera from patients infected with *O. volvulus* (Klenk *et al.* 1984). These demonstrations of circulating antigens in patients with filariasis suggest an alternative approach to immunodiagnosis. Instead of developing methods to detect specific antibodies in the blood, detection of specific antigen should be possible.

The presence of a protease on *D. immitis* was first demonstrated by Sato, Takahashia and Swada (1976). Later Sato *et al.* reported that two potentially purified globin hydrolysing "enzyme fractions" from these parasites could serve as parasite specific antigens for immuno-diagnosis. Ott, Staples, Weekley and Maggio (1985) demonstrated both immunologically unique and common antigenic determinants in *Dirofilaria immitis* and *Toxocara canis* using monoclonal antibodies. Various methods of radioiodination were employed to identify peptides on the surface of *D. immitis* microfilaria by Tamashiro, Ehrenberg, Levy and Scott (1986). Optimum surface radiolabelling occurred with the lacto-peroxidase-catalase reaction. Two major peptides of 16 and 14 KD were labelled by this method.

Accurate diagnosis depends not only on a standardised and characterised antigen, but also on a suitable assay system. Assays should be sensitive, specific, quantitative, economical and suitable for use in the endemic areas. The most sensitive serological assay systems, radioimmunoassay and Elisa, have been applied in experimental diagnostic tests for nematode infections (Ouassi *et al.* 1981; De Savigny *et al.* 1979; Maizels *et al.* 1984; Van Knapen *et al.* 1980).

The ability to clone the gene which produce the diagnostic antigen and then introduce it into suitable expression systems would allow almost limitless amounts of antigen to be prepared relatively easily and cheaply. The study of parasitic nematode antigens has illuminated the subtle nature of the interaction between parasite and host. Though there is a cell mediated response due to the invading larvae, it is not pertinent to this thesis and will not be discussed here. It has been reviewed by Sugane and Oshima (1980, 1982).

So far, functions of the parasitic antigens have been ascribed to only two nematode parasite antigens, and both were found to be enzymes. The first demonstrated was the acetyl cholinesterase of *N. brasiliensis* (Jones and Ogilvie 1972; Sanderson *et al.* 1972). This has been postulated to act as a biochemical holdfast for the worms in the gastrointestinal tract (Phillip 1984). Furthermore, it has been shown that worms adapted to survive in an immune environment produce enzymes of different electrophoretic mobility (Edward *et al.* 1971). One may envisage that acetyl cholinesterase activity is essential for parasite survival and that the enzyme, released by worms adapted to the immune environment, is not inactivated by host antibodies. The second

example of an antigen whose function is known is the superoxide dismutase of *Trichinella spiralis* (Rhoads 1983). This enzyme, secreted by infective larvae, is identical to bovine superoxidase (a dimer of 36 KD), and yet dismutase displays antigenic determinants recognised specifically by sera from animals infected with *Trichinella*. Once again a biological role has been proposed for this enzyme, protecting the parasite from the lethal products of the host is cellular defense and thus specific antibodies could protect the host (Rhoads 1983). Chen and Howells (1979) demonstrated that the cuticle of adult *Brugia pahangi* transports specific nutrients into the worm for metabolic use. To be able to transport components actively across the cuticle, the worm requires transport proteins inserted into the surface and receptors freely available to the external medium. These molecular structures, vital to worm survival, could also be targets for host responses. Not all antigens of nematodes need mediate essential metabolic functions for the worm. An alternative reason for their continued exposure is that they actually allow the parasite to survive by stimulating a host response. Second stage larvae of *Toxocara canis* release vast amounts of secretions derived from the surface which can prevent the binding of antibodies to the parasite's surface (Smith *et al.* 1981). In addition, the constant exposure and shedding of these antigenic components may divert the attention of the immune system away from other molecules vital to host survival.

Finally, the stimulation of responses to stage-specific antigens may prevent secondary, life threatening infections of the host. For example, the infective larvae of *T. spiralis* in the muscle secretes a strongly immunogenic glycoprotein which has been shown to be host protective (Silberstein and Despommier 1984). This same secretion is released during a normal infection (Capo *et al.* 1985). Thus the

larvae could prevent the establishment of a second potentially lethal infection in the gastrointestinal tract, thus ensuring the survival of established parasites.

A problem related to the parasite maintained in the laboratories is that the worms get so well adapted to the laboratory environment that they may even contain some enzymes of altered electrophoretic mobility (Edwards *et al.* 1971), and survive for prolonged periods in the gastrointestinal tract of otherwise immune animals (Jenkins and Philipson 1972). Their survival in the host is not due to a different antigenic structure of the surface of the worms (Maizels *et al.* 1983a) and therefore may be due to a change in the responsiveness of the host rather than a change in the worms.

Despite these doubts, the successes of recent work by Ortega-l'ierres *et al.* (1984), Philip, Gomez-Priego, Parkhouse, Davies, Clark and Ogilvie 1984; Meghiji and Maizels (1986) shows promise that specific and genetically engineered antigens will be highly useful for detection of many nematode infections.

CHAPTER 2

MATERIALS AND METHODS

2.1 PARASITE MATERIAL

Adult *T. canis*, *T. cati*, *T. leonina* and *D. immitis* were recovered from either dogs killed at the Canberra and Queanbeyan Dog Pounds, or cats from the Canberra RSPCA. Adult *A. suum* were obtained from pigs slaughtered at Cootamundra, Gundagai and Junee abattoirs. *D. roemeri* were recovered from kangaroos which were culled at the Tidbinbilla Nature Reserve in the Australian Capital Territory.

2.2 PRODUCTION OF ES ANTIGENS FROM ADULT WORMS

The above intestinal parasites were thoroughly washed in running tap water and transferred to 0.85% normal saline solution (NSS). They were washed twice with sterile NSS and finally with NSS containing 100 units/ml penicillin and 100 mg/ml streptomycin (Glaxo), and transferred to 200 ml of RMPI culture media. The culture media was gassed with 5% CO_2 in screw capped bottles and incubated at 37°C. Phenol red in the culture medium was used to monitor pH changes and to indicate equilibration with CO_2. The liquid to air ratio was maintained at 1:7.

RMPI Culture Media (1640)

Components	mg/litre
L-arginine	200.0
L-asparagine H_2O	56.82
L-aspartic acid	20.00
L-cystine, disodium salt	59.15
L-glutamic acid	20.00
L-glutamine	300.0
Glutathione	1.00
Glycine	10.00
L-histidine	15.00

RMPI Culture Media (1640) (cont.)

Components	mg/litre
L-hydroxyproline	20.00
L-isoleucine	50.00
L-lysine HCl	40.00
L-methionine	15.00
L-phenylalanine	15.00
L-proline	20.00
L-serine	30.00
L-threonine	20.00
L-tryptophan	5.00
L-tyrosine disodium salt	24.86
L-valine	20.00
Biotin	0.20
D-calcium pantotherate	0.25
Choline chloride	3.00
Folic acid	1.00
I-inositol	35.00
Nicotinamide	1.00
P-aminobenzoic acid	1.00
Pyridoraine HCl	1.00
Riboflavin	0.20
Thiamin HCl	1.00
Vitamin B_{12}	0.005
$Ca(NO_3)_2$	69.49
KCl	400.0
$MgSO_4.7H_2O$	100.0
NaCl	6000.0
Na_2HPO_4	800.7
D-glucose	2000
Phenol red sodium salt	5.0

RMPI 1640 with glutamine and without $NaHCO_3$ was obtained from Flow Laboratories (Australasia, North Ryde NSW). A sachet containing 10.38 grams was dissolved in glass distilled water containing 0.3-0.4 ml of concentrated HCl (AR grade). Dry $NaHCO_3$ was added to this solution on a magnetic stirrer until it reached a pH range of 6.9-7.05. This solution was sterilized by passing it through a sterile microfilter and the pH was adjusted by adding sterile 1.4% $NaHCO_3$ solution. 100 units/ml penicillin and 100 µg/ml streptomycin were added to the solution prior to use.

2.2.1 Extraction of Surface Antigens

Another batch of the same parasites was cleaned and agitated in a solution containng 0.1% Tris (Hydroxyl-methyl aminomethane) and 1% deoxycholate on a Vortex agitator for 1/2 an hour to extract the cuticular antigens.

Eggs laid during the first 24 h of the culture were collected, washed in distilled water and allowed to embryonate at room temperature. More eggs were obtained by dissecting gravid females at the end of culture. These eggs were hatched by Fairbairn's method (Fairbairn 1961). Spent culture media was centrifuged and the supernatant frozen till required.

2.3. PREPARATION AND HATCHING OF EGGS

Eggs embryonated at room temperature were examined periodically for larval development. It took 3-4 weeks for these eggs to show fully developed stage two larvae. Eggs containing viable, fully developed second stage larvae were considered ready for hatching.

Three ml of a dense suspension of embryonated eggs were treated with 0.1 N H_2SO_4 at 37°C overnight. This was transferred to a sterile

conical flask with a screw cap top. The chitinous shell was removed by suspending the eggs in sodium hypochlorite solution (fresh undiluted household bleaching solution containing about 6% available chlorine was best for this purpose) and shaking for 4 hr at 35°C. A drop of the suspension was heated at 80°C and when examined under the microscope contained only free larvae and globules of the melted vitalline larvae membrane. Following 3-4 weeks in distilled water, this sterile suspension was transferred into four test tubes with screw caps for hatching.

2.3.1 Hatching Media

To the sterile suspension of 1.5 ml of eggs, sterile sodium chloride (0.25 M) + 0.0025% polyoxyethylene sorbitan mono-oleate (Tween 80), 0.04 M sodium hydrosulphite (Dithionite) and 1.0 ml of pregassed 0.1 M sodium bicarbonate were added. These tubes were gassed for 15 minutes at 35°C with N_2:CO_2 (95:5)% and incubated at 38°C for 5 h in a water bath. About 90% of the eggs hatched.

Eggs of different species were treated according to the type of the egg shell membrane. Eggs with thick shell membranes needed acid treatment whereas others needed only a mechanical interference. *T. cati* and *T. pteropodis* egg shell membranes were not as thick as *T. canis* and thus the eggs did not need any acid treatment, the rest of the procedure was the same.

2.4 *IN VITRO* CULTURE OF INFECTIVE LARVAE (de Savigny 1975)

The larval suspension obtained by Fairbairn's method (Fairbairn 1961) was washed three times in Hanks Balanced Salt Solution (BHSS) with 100 units/ml penicillin and 250 µg/ml streptomycin and held overnight in HBSS at 37°C in a Baermann apparatus consisting of fine nylon gauze. Most viable hatched larvae migrated through the gauze while unhatched eggs, hatching debris and dead larvae were retained in the gauze.

2.4.1 Production of ES Antigens from Larval Culture

The larvae collected following Baermann separation did not require further washing and were dispersed immediately into a simple defined Eagle's Minimal Essential Medium with Hank's salts (HMEM) containing 100 units/ml penicillin and 250 µg/ml streptomycin (Flow Laboratories, Australasia, North Ryde NSW) in Leighton tubes. The tubes were incubated at 37°C. Every 7th day tubes were examined for contamination or mortality. Any tubes showing contamination and any tubes in which mortality exceeded 5% were discarded. In satisfactory tubes the larvae were allowed to settle and the spent medium was aspirated aseptically. Fresh volumes of sterile medium were added to the tubes using a sterile syringe and the tubes were returned to the incubator. *T. canis* larvae survived for over 20 months in this medium. The spent medium was pooled and centrifuged to remove any larvae which may have been aspirated.

Hanks balanced salt solution:

Component	mg/l
NaCl	8000.0
KCl	400.0
Na_2HPO_4	47.50
KH_2PO_4	60.0
$MgSO_4.7H_2O$	200.0
$CaCl_2\ 2H_2O$	185.5
Glucose	1000.0
$NaHCO_3$	350.0
Phenol red sodium salt	17.0

Eagle's Minimum Essential Medium with Hanks Salts:

	mg/l		mg/l
L-arginine HCl	126.4	NaCl	8000.0
L-cystine disodium salt	28.42	$NaHCO_3$	350.0
L-glutamine	292.3	$NaH_2PO_4.2H_2O$	2583.0
L-histidine HCl H_2O	41.90	Na_2HPO_4	47.50
L-isoleucine	52.50	Dglucose	1000.0
L-leucine	52.50	Phenol red sodium salt	17.00
L-lysine HCl	73.06		
L-methionine	14.90		
L-phenylalanine	33.02		
L-threonine	47.64		
L-tryptophan	10.20		
L-tyrosine disodium salt	45.02		
L-valine	46.90		
D-capanthotherate	1.00		
Choline chloride	1.00		
Folic acid	1.00		
I-insotol	2.00		
Nicotinamide	1.00		
Pyridonal	1.00		
Riboflavin	0.10		
Thiamine HCl	1.00		
$CaCl_2.2H_2O$	185.5		
KCl	400.00		
KH_2PO_4	60.0		
$MgSO_4.7H_2O$	200.0		

Embryonated eggs of *T. pteropodis* were obtained from Dr P. Prociv of the Queensland University. The eggs were received on charcoal, from which they were separated by eluting vigorously in water and separation by sedimentation and filteration.

2.4.2 *In vitro* Culture of Dirofilarial Parasites

Adult *Dirofilaria roemeri* and *D. immitis* were recovered from the heart of dogs and under the sartori muscles of kangaroos, respectively. They were washed in sterile saline solution (0.85%) five times and cultured in RMPI at 37°C for 24 h. The culture was checked every 6 h to check for contamination. Following 24 h the adult worms were removed for extraction of surface antigens. The culture material was allowed to stand so as to let the microfilariae settle. The supernatant was removed by aspirating it out aseptically for antigen purification. Purified antigens were divided into small aliquots and frozen at -70°C until required.

2.4.3 *In vitro* Culture of *Angiostrongylus cantonensis*

Infective third stage larvae were obtained from Dr John Walker of the Institute of Tropical Medicine, Sydney. These larvae were washed in a snail saline-Hedon-Feleig salt solution.

Hedon-Feleig salt solution:

Component	gm/litre
NaCl	7.0
KCl	0.3
CaI_2	0.1
$MgSO_4.3H_2O$	0.3
$NaHCO_3$	1.5

Distilled water was added to give a final volume of 1 litre, pH 7.96, (van Noordyde and de Wolf 1963).

While giving the final wash, larvae were suspended in Hanks salt solution containing 100 iu/ml benzyl penicillin and 100 µg/ml streptomycin in 1/2-1 h. This incubation showed if there was any contamination by the change in colour of the culture due to change in pH. Larvae were divided into batches to culture them at 37°C and 20°C so as to observe the effects of temperature on antigen production. Larvae which were to be biosynthetically labelled were transferred aseptically to methionine free Eagles media for 12-24 h.

2.5 MAINTENANCE OF LIFE CYCLE

Angiostrongylus cantonensis has an intermediate host, for example snails or crustaceans. The snails used for infection were received from the Institute of Tropical Medicine, Sydney, where a colony of *Isidorella newcombi* is maintained, derived from the Hugh River, of the Macdonnell Ranges, Northern Territory. These snails were maintained on the following snail food: lettuce, wheat germ and lucerne.

Lettuce leaves were dried in an oven until brittle (not cooked and brown), lucerne was cut and dried without the stems. Equal parts of each was placed in a blender and blended to a fine powder. A few marble chips were added to the tank. Snails to be infected were starved for 24 hrs and exposed to rat stools infected with first stage larvae. This was best carried out by feeding snails with snail food smeared with infected rat stools.

2.5.1 Harvesting of Third Stage Larvae

One month after infection, fully developed third stage infective larvae were obtained from the snails. Snails were starved for 24 hr and transferred to sterile water so as to minimise the contamination. They were washed in 0.05% Hibitaine prior to dissection.

Infective third stage larvae were obtained by opening the haemocoel and tissues of the snail. All the tissues except the alimentary tract and the gonads were cut into pieces and Baermannised overnight at room temperature in Hedon-Fleig salt solution. The funnel had a thin layer of cotton wool gauze sandwiched between a layer of Kim-wipe paper. This was held by a nylon mesh. Good results were obtained by harvesting the larvae within 15 h. These larvae were either washed and cultured for antigen production or used to infect rats and mice in order to maintain their life cycle and produce antisera respectively.

Adult *A. cantonensis* were obtained from the pulmonary vessels of the rat to study the adult cuticular extracts of the parasite. Snails infected with first stage larva and rats infected with third stage larvae were maintained in the Animal House to continue their life cycle.

2.6 PRODUCTION OF NORMAL AND INFECTED SERA

T. canis, T. cati, T. pteropodis, A. suum. Second stage larva were used to infect batches of five CB mice for each parasite. Five hundred larvae per mouse were used in this group and 20 *A. cantonensis* third stage larva were used or infecting the mouse with *A. cantonensis* third stage larvae. Sera were collected after a minimum period of 5 weeks. [The handling and use of these animals followed to the Guidelines set out in the Code of Practice for the Care and Use of Animals for Experimental Purposes].

These mice were deeply anaesthetised and an incision was made in the skin across the ventral side of the thorax. The skin was folded back exposing the axilla on one side and the subclavian artery and vein were severed. The blood which was collected in the axilla was quickly transferred by a Pasteur pipette to a glass centrifuge tube. The blood was allowed to clot at room temperature for about 60 minutes before

freezing the clot from the tube. The blood was kept at 4°C overnight and then centrifuged at 980 rpm for 20 min. The supernatant was recentrifuged for 30 min and the serum divided into small aliquots for storage at -20°C until used. The same procedure was carried out for batches of uninfected mice under the same conditions. Sera from these animals were used as Controls.

2.6.1 Human Sera

T. canis and *A. cantonensis* positive human sera were obtained from clinical cases referred to the Commonwealth Institute of Health in Sydney. Negative serum was chosen from several hundred sera, obtained from healthy blood donors which had been tested for respective parasite antibodies as described by Nicholas, Stewart and Walker (1986).

2.7 ANTIGEN PURIFICATION

Antigens were extracted from two sources: (a) adult or larval cultures (E/S) and (b) surface extraction using detergents. Antigen extracts were centrifuged at 3000 rpm for 20 min at 4°C and the supernatant dialysed against M/15 phosphate buffered saline at pH 8 with 0.01% sodium azide in the solution as preservative.

Preparation of Dulbecco's Phosphate Buffered Saline

The following three solutions were prepared.

Solution A		Solution B		Solution C	
Compound	Weight	Compound	Weight	Compound	Weight
NaCl	8 g	$CaCl_2$	0.1 g	$MgCl_26H_2O$	0.1 g
KCl	0.2	Made up to 100 ml		Made up to 100 ml	
Na_2HPO_4	1.15				
KH_2PO4	0.2				
Made up to 80 ml					

Each solution was prepared separately and then mixed.

Dialysis tubes were cut into convenient lengths (10-20 cm), boiled for 10 min in a large volume of 2% sodium carbonate and 1 mM disodium Ethylene diaminetetra acetate (EDTA). They were thoroughly rinsed in distilled water, boiled for 10 min in 0.001 M EDTA, allowed to cool and stored at 5°C well submerged. The tubing was handled with gloves and was washed inside and out with distilled water before use. Antigen extracts were transferred into these tubes and dialysed for 72 h at 4°C with 4-5 changes of PBS. Initial changes were made frequently so as to remove the impurities quickly. Antigens were then concentrated by dialysis against 30% polyethylene glycol (Aquacide III, Calbiochem, Sydney) or Amicon concentrations (Amicon Corp, Danvers, USA). Finally the purified antigens were divided into small aliquots and frozen at -70°C until required.

2.8 **PROTEIN DETERMINATION** (Bradford 1976)

Protein determination utilised the binding of protein to Coomassie Brilliant Blue G-250, causing an increase in absorbance at 620 nm. The protein reagent comprised 100 mg of Coomassie Brilliant Blue G-250 (Sigma) dissolved in 50 ml 95% ethanol. To this was added 100 ml of 85% orthophosphoric acid and the solution was made up to one litre with distilled water: 150 µl of the protein reagent was used for the assay. 50 µl aliquots of suitable dilutions of protein sample were added and absorbance read in a Titertek Multiskan MC Spectrophotometer (Flow Laboratories). Protein concentration was determined by reading from a standard curve which was linear up to a concentration of 0.05 mg of protein/ml. Bovine serum albumin (Commonwealth Serum Laboratories) was used as the standard.

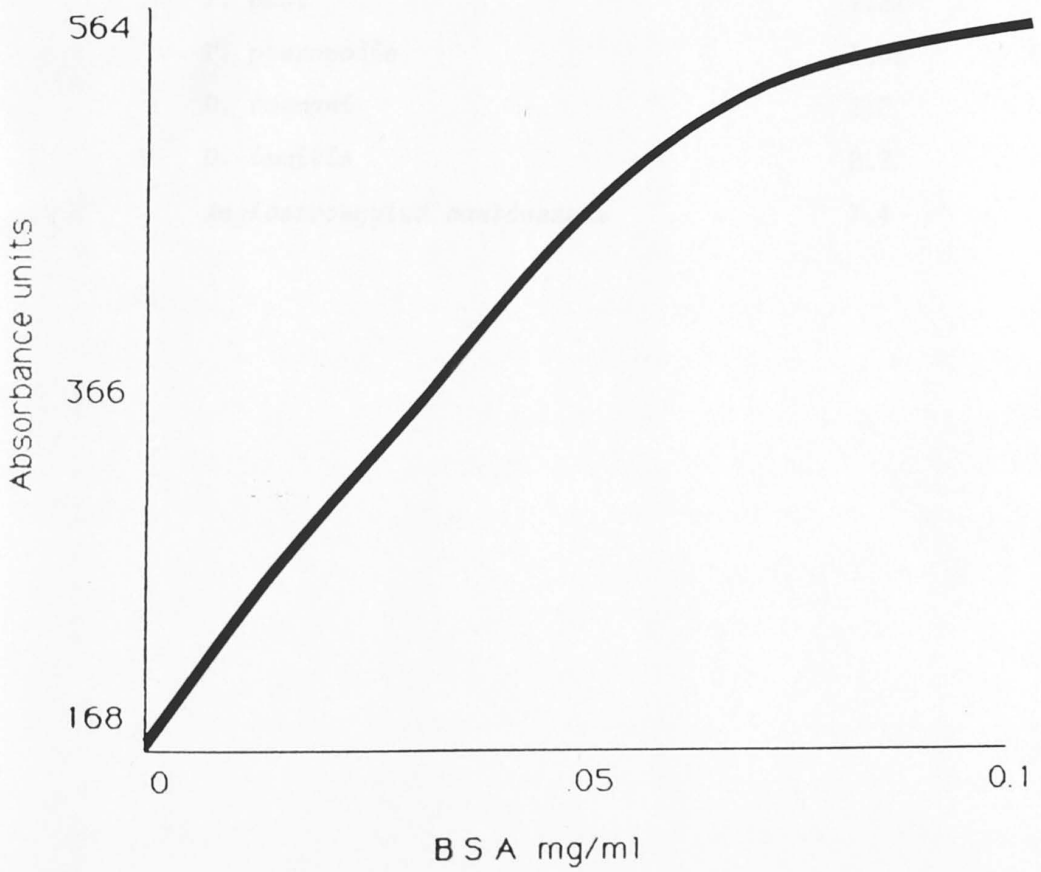


Fig. 2.1 Standard curve for protein determination

Table 2. Protein determination of parasite antigens.

Parasite	Quantity of protein in µg/ml
T. canis	1.33
T. cati	1.22
T. pteropodis	1.45
D. roemani	2.2
D. immitis	2.3
Angiostrongylus cantonensis	7.4

CHAPTER 3

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (ANTIGEN)

3.1 INTRODUCTION

Polyacrylamide gel has potentially high resolution because the degree of molecular sieving can be controlled by the concentration of the gel. The versatility of this medium is shown by the variety of electrophoretic techniques which make use of polyacrylamide gels. The original disc electrophoretic technique (Davis 1964) has the disadvantage that samples must be compared in separate gels. However, this problem has been overcome by the use of polyacrylamide slab gels. The resolution of this media has been improved by the introduction of techniques such as SDS-polyacrylamide gels with continuous (Weber and Osborn 1975) and discontinuous buffers (Laemmli 1970); polyacrylamide gradient gels (Johnson 1977), isoelectric focusing (Righetti 1983) and two dimensional gels (O'Farrell 1975; O'Farrell *et al.* 1977). Techniques using SDS are not generally suitable for enzyme electrophoretic studies because the detergent denatures the protein. However methods are available to renature the proteins after electrophoresis (Lacks and Springhorn 1980). The main disadvantages of the acrylamide are the neurotoxicity of the unpolymerized components and occasional problems with polymerization of the gel.

Polyacrylamide gels are generated by the free radical polymerization products of the acrylamide monomer (CH_2 = CH-CO-NH_2) and the cross-linking co-monomer N,N'-methylene-bis-acrylamide (CH_2 = CH-CO-NH-CH_2-NH-CO-CH = CH_2). The polymerization reaction is initiated by a catalyst redox system which furnishes free radicals (Maurer 1971; Gordon 1975). The most commonly used system utilizes the tertiary amine

TEMED (N,N,'-N'-tetramethylethylenediamine) as the catalyst and amonium persulphate as initiator. Treatment of proteins with the detergent SDS (sodium dodecylsulphate) and a reducing agent (beta-mercaptoethanol) changes their three dimensional shape into rod-like structures (Reynolds and Tanford 1970). Since SDS binds to polypeptides at a constant weight ratio the charge per unit weight is constant and the electrophoretic mobility becomes a function of molecular weight (Reynolds *et al.* 1970). In addition to protein separation, the SDS-polyacrylamide gel electrophoresis technique has been widely used to determine the molecular weight of unknown proteins by comparing their relative electrophoretic mobility (Rf) to standard proteins of known molecular weights (MW) (Weber and Osborn 1969; Gordon 1975). A straight line is obtained in a plot of log MW as a function of Rf (Fig. 3.1).

Several systems for SDS-polyacrylamide gel electrophoresis have been described. This Chapter describes the discontinuous system introduced by Laemmli (1970) for disc gel electrophoresis, which was later adapted to slab gels by Studier (1973). The system is characterised by a discontinuity in the buffer pH and in the polyacrylamide pore size. Two kinds of gel are prepared: a "stacking gel" with large pores at pH (6.8) where the sample is concentrated, and a "resolving gel" ("running gel") with small pores at a more basic pH (8.8) where the sample is separated into its components. The pore size of the running gel can be adjusted according to the molecular weight heterogenicity of the polypeptides in the sample; high concentrations of acrylamide (10-12%) give better separation of low MW proteins while low concentrations of acrylamide (5-6%) favour the separation of higher MW proteins.

This Chapter will describe the preparation of homogeneous 10% SDS-polyacrylamide slab gels and the staining techniques for protein visualization.

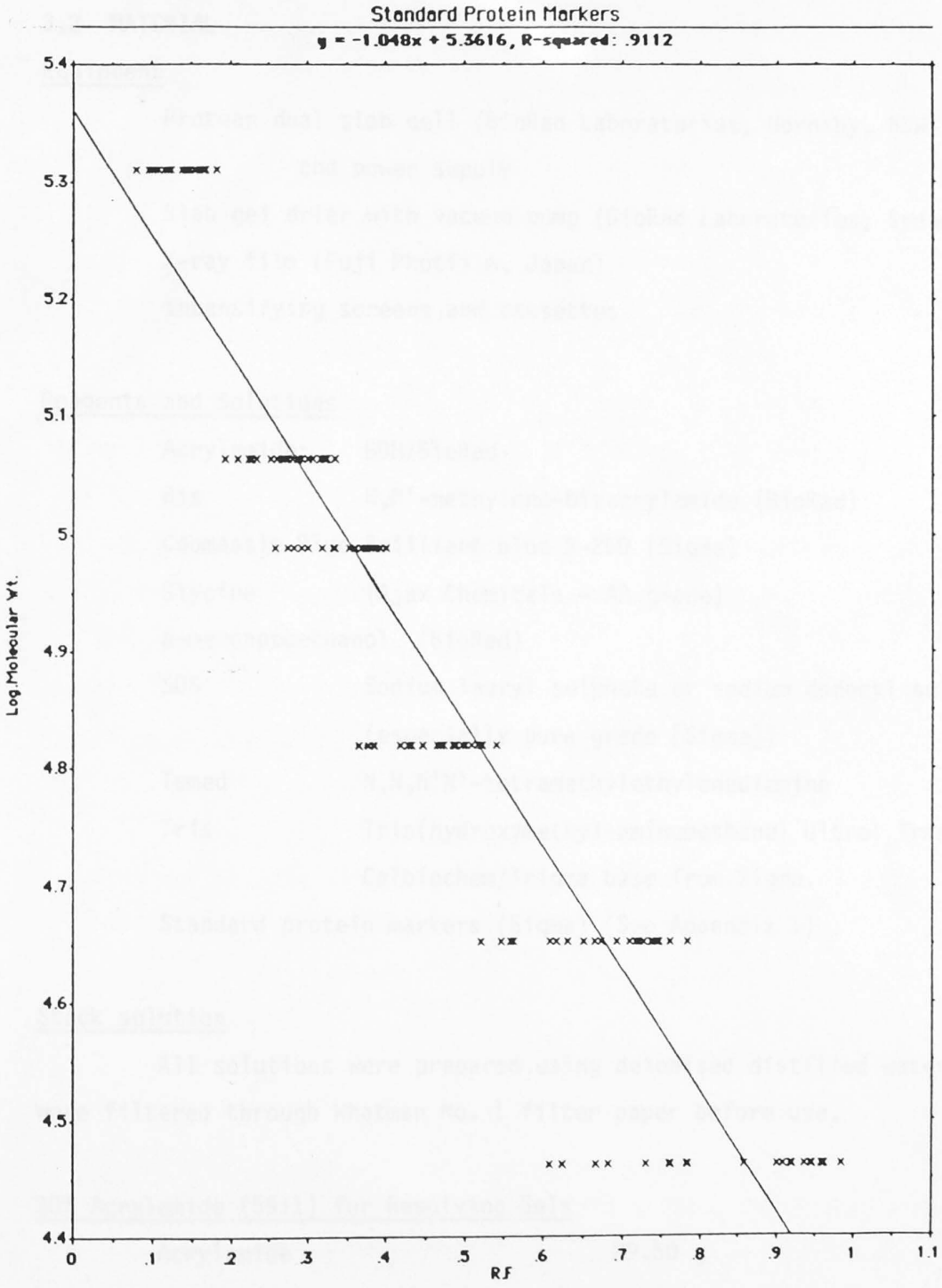


Fig. 3.1 Calibration for determining the molecular weight of polypeptides after electrophoresis on SDS polyacrylamide gels. (Same protein markers from 25 gels were standardised: see Fig. 4.2).

3.2 MATERIAL

Equipment

Protean dual slab cell (BioRad Laboratories, Hornsby, NSW)
and power supply

Slab gel drier with vacuum pump (BioRad Laboratories, Sydney)

X-ray film (Fuji Photfilm, Japan)

intensifying screens and cassettes

Reagents and Solutions

Acrylamide:	BDH/BioRad
Bis	N,N'-methylene-bisacrylamide (BioRad)
Coomassie Blue	Brilliant blue R-250 (Sigma)
Glycine	(Ajax Chemicals - AR grade)
β-mercaptoethanol	(BioRad)
SDS	Sodium lauryl sulphate or sodium dodecyl sulphate (especially pure grade [Sigma])
Temed	N,N,N'N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl-aminomethane) Ultrol Tris from Calbiochem/Trigma base from Sigma.

Standard protein markers (Sigma) (See Appendix 1)

Stock solution

All solutions were prepared using deionised distilled water and were filtered through Whatman No. 1 filter paper before use.

30% Acrylamide (59:1) for Resolving Gels

Acrylamide	29.50 g
Bis	0.50 g
Water	100 ml

Dissolved acrylamide before Bis made up to 100 ml with water and stored in a brown bottle at 4°C.

30% Acrylamide (36:1) for Stacking Gels

Acrylamide	29.20 g
Bis	0.80 g

Water to 100 ml and stored at 4°C in a dark bottle.

4 x Resolving Gel Buffer

Tris-HCl	45.425 g (1.5 M)
SDS	1.0 g (0.4%)

Made up Tris to about 200 ml, added SDS and, when dissolved, adjusted pH to 8.8 using 6 N HCl made up to 250 ml with water, filtered and stored at room temperature.

4 x Stacking Gel Buffer

Tris-HCl	15.15 g (0.5 M)
SDS	1.0 g (0.4%)

Prepared as for resolving gel buffer, but adjusted pH to 6.8 with 6 N HCl made up to 250 ml, filtered and stored at room temperature.

10 x Reservoir Buffer

Tris	30.2 g (0.25 M)
Glycine	144.0 g (1.92 M)
SDS	10.0 g (1%)

Dissolved Tris first in 800 ml water, added glycine and heated gently to dissolve before adding SDS which also needed a little heat to dissolve. Checked pH which should be 8.3. To avoid too much variation in ionic strength care was taken in adjusting the pH. Made up to 1 litre and stored at room temperature.

10% Ammonium Persulphate

Ammonium persulphate	1.0 g

Made up to 10.0 ml with water. A pure salt is identified by the vigorous effervescence on adding the water. Fresh batches of ammonium persulphate are always recommended and a solution can be stored at 4°C for at least a month.

2 x Sample Buffer

4 x stacking gel buffer	25.0 ml (0.125 M)
10% SDS	60.0 ml (6%)
Glycerol	20.0 ml (20%)
Bromophenol blue (0.4%)	

Stored at room temperature. 100 µl of β-mercaptoethanol was added to 0.9 ml 2 x sample buffer before use.

Staining Solution

Coomassie Blue	1.0 gm (0.20%) in
Methanol:acetic acid:water	(5:1:5)

Destaining Solution

Ethanol	600 ml
Acetic acid	250 ml
Water	1750 ml

3.3 METHOD

3.3.1 Assembly of the Slab Gel Apparatus

The slab gel was prepared between two glass plates. The plates were perfectly clean. They were left in a concentrated detergent solution for two hours, washed thoroughly with water and then with ethanol. The dried plates were placed together with the notch at the top and the glass spacers between them along the side edges. The two

glass plates and the spacers were tightly assembled and the whole system was tightened with clamps. The plates were placed vertically on a flat surface.

3.3.2 Preparation of Resolving Gel (10%)

This was prepared the day prior to use so as to allow for complete polymerization of the acrylamide.

Water	12.5 ml
4 x resolving gel buffer	7.5 ml
30% acrylamide (59:1)	10.0 ml
Temed	10 µl
10% ammonium persulphate	60 µl

Solutions were mixed without Temed. As soon as the Temed was added up to about 2.5 cm below the notch, the resolving gel was rapidly poured between the glass plates. The gel was then gently overlayed with butanol saturated with water in order to achieve an even surface. Polymerization occurs within 30-60 min at room temperature.

The liquid above the gel was poured off and 1 x resolving gel buffer was added (ie. 4 x resolving gel buffer diluted 1:3 with water). The gel was left for 10 minutes before pouring off and overlaying buffer was replaced with fresh 1 x resolving gel buffer. The mouth of the glass plates were sealed with parafilm and was left to stand overnight.

3.3.3 Preparation of Stacking Gel (5%)

The stacking gel was always prepared on the day of use and no more than 2 hr before running the gel.

The overlay buffer was removed from the resolving gel and replaced by 1 x stacking gel buffer. This was rinsed once, then more buffer was added and left while preparing the stacking gel solution. The stacking gel was prepared by mixing:

Water	5.84 ml
4 x stacking gel buffer	2.5 ml
30% acrylamide (36:1)	1.66 ml
Temed	5 µl
10% ammonium persulphate	100 µl

The stacking gel buffer was poured off and the last traces were removed with filter paper, making sure not to touch the gel surface with the filter paper. The comb was carefully inserted from one end first to ensure no air bubbles became trapped beneath the teeth. Polymerization was completed within 30 min on the bench.

Following polymerization the comb was removed which made fifteen equal size wells. These wells were rinsed with 1 x reservoir buffer to remove any unpolymerized acrylamide and loaded with the samples under reservoir buffer as explained below.

3.3.4 Antigen Sample Preparation for Electrophoresis

These samples were prepared while stacking gel was polymerizing. 100 µl of β-mercaptoethanol was added to 0.9 ml 2 x sample buffer. A volume equivalent to the sample volume was then added to each antigen sample and boiled in closed capped Eppendorf tubes which were held in a boiling water bath for 1.5 min.

3.3.5 Electrophoresis

The Protean Dual slab cell (BioRad) consists of an upper chamber and a main reservoir tank. The clamped glass plates with gel were fixed to this upper chamber and secured properly using the Delrin

cams. Samples were loaded with a Gilson pipette fitted with a Drummond Micro capillary tube. At maximum volume of 100 µl can be loaded on to a single well. Care was taken so as not to overload the wells. Following the loading of samples, the upper chamber fitted to the loaded gels were removed from the setting stand and were transferred and fixed into the main reservoir. Both the upper chamber and the reservoir tank were sufficiently filled with reservoir buffer. The power supply was adjusted to give a constant current but voltage was allowed to vary. Electrophoresis was started at 30 mA until samples had run into the gel and then increased to 40 mA. Standard molecular weight markers were always run on each gel. Electrophoresis was carried out until the bromophenol blue tracer had migrated almost to the end of the running gel. At the end of the run, the plates were removed from the electrophoresis chamber. The side spacers and the glass plates were removed with a spatular and the gels transferred to a dish containing stain.

3.3.6 Staining and Destaining

The gels were stained in Coomassie blue for 1/2 hr at 37°C or 60 min at room temperature. The stain was aspirated out and replaced with the destaining solution. Gels were agitated gently on a rotary shaker for 10 min and the destaining solution was replaced again and left overnight. Frequent change of destaining resulted in rapid destaining. Clear appearance of standard protein was indicative of perfect destaining. Clear gels were obtained by soaking the gels in 7% (Ag) acetic acid for 30 min. Photographs of gels were taken with a Polaroid camera using diffused light (Fig. 3.2).

3.3.7 Drying of Gels

A 3 mm filter paper was cut slightly longer than the gels. The filter paper was immersed into the dish and under the gel, so as to

float the gel over the paper. Once the gel was positioned the filter paper was lifted gently out of the solution with the gel attached to it. The wet gel with the filter paper was placed in the following order on the gel slab drier: gel drier, mesh screen, porous white polyethene sheet, 3 mm paper, 3 mm paper with gel attached, and upper most layer of transparent mylar sheet wrapped in Glad Wrap. The drier was connected to a good vacuum source. The gel and other supportive material were covered with a rubber sheet to maintain the suction. The drier was switched on with the timer set for 1 hr. The gel was allowed to cool for another 2 h while suction was on. Once the gel was dried perfectly the vacuum was broken by lifting the rubber. The dried gel was removed carefully and it was sealed in a plastic bag to prevent it from curling.

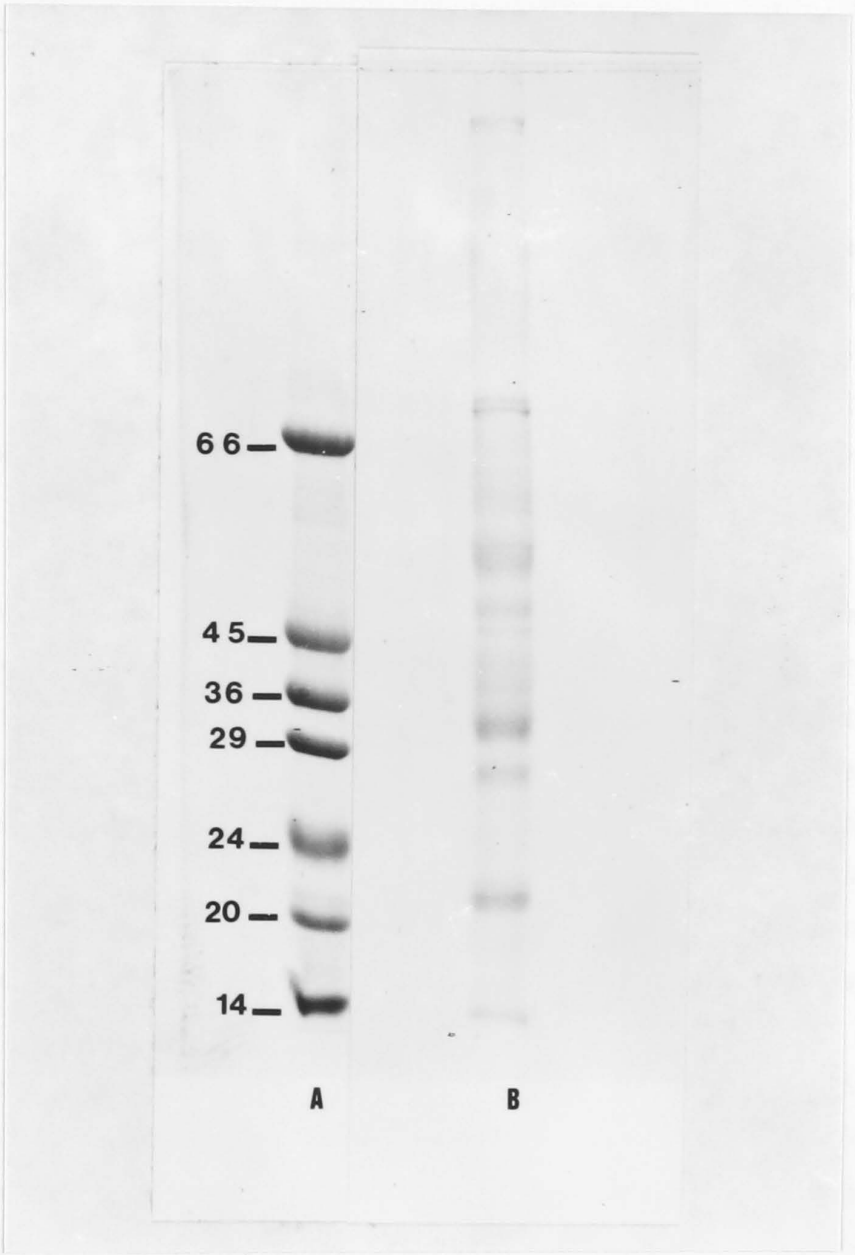


Fig. 3.2 Coomassie blue stained SDS PAGE gel. Lane (A) standard protein molecular weight markers; Lane (B) ES antigens extracted from *in vitro* culture of adult *Toxascaris leonina*

CHAPTER 4

RADIO LABELLING OF ANTIGENS

4.1 INTRODUCTION

The surface of living nematode worms can be radioiodinated with ^{125}I without loss of viability, and subsequently the labelled molecules can be recovered in a soluble form (Parkhouse, Phillips and Ogilvie 1981), using a technique originally developed for labelling protein solutions (Greenwood, Hunter and Glover 1963; Bolton and Hunter 1973).

This is a finding of some significance, since the cuticle of the nematode is remarkably different from the mammalian cell membrane, and indeed from the tegumental coverings of other helminths (Lee 1984, 1972; Lumsden 1975; Bird 1980). Despite its acellular composition and multilaminar membranous boundary, quite different in appearance from a plasma membrane, the cuticle can be approached biochemically by techniques transferred from mammalian studies. Another important difference is the apparent impermeability of most nematode cuticles to even low molecular weight substances (Pappas and Read 1975; Chen and Howells 1979), which permits the surface labelling of nematodes with reagents which could penetrate a cell plasma membrane. In addition to its biological interest, this technique has important ramifications for the immunological analysis of nematode infections. Table 4.1 summarizes the principal advantages of using surface labelling, firstly as an analytical method, and subsequently using labelled surface molecules to delve further into immunity in the host-parasite relationship.

A variety of methods are available for the radioiodination of proteins, suggesting that no single method has been found to be completely satisfactory.

Table 4.1 Surface labelling as an approach to the problems of nematode immunology.

Identification of Antigens	With a restricted number of surface labelled proteins, each can be identified and assessed for antigenicity.
Purification of Antigens	Selective labelling of the surface molecules, combined with radioactive assays, may make antigen purification unnecessary. Labelled molecules can be directly analysed biochemically.
Scarcity of Material	The Amplification factor of radioiodination is very large and minute quantities of material may be detected.
Assay of Immune Responsiveness	Antibody levels, and specificity of antibody can be directly measured with surface labelled antigens. These antigens can be used to develop serological methods for immunodiagnosis. Immunoprecipitate experiments using *Staphylococcus aureus* and Western blot are described in the Chapters to follow.
Molecular Comparison of Nematodes	Immunological and biological relationships between different stages of the nematode life cycle and between different nematode species can be analysed by SDS PAGE gel electrophoresis.

Table 4.1 (cont.)

Study of the Nature of the Nematode Surface	Surface labelled molecules are ideal probes for the analysis of dynamic properties of the surface and as tracers in biochemical investigations of the cuticle. (Modified from Maizels, Philipp and Ogilvie 1982).

An important technical point is the bias of these surface-labelled techniques towards polypeptides containing lysine and tyrosine residues. Polysaccharide and glycolipid antigens, for example, may escape labelling altogether and consequently be excluded from analyses.

4.1.1 **Radioiodination - Chloramine-T Method** (Parkhouse, Phillip and Ogilvie 1981)

This is the most widely used method for the radioiodination of small amounts of protein at high specific radioactivity for use as tracers in radioimmunoassays. $Na^{125}I$ is oxidised by chloramine-T in the presence of the protein to be labelled, with the subsequent incorporation of ^{125}I into the tyrosine residues of the protein in high yield. Excess chloramine-T is reduced by the addition of sodium metabisulphite and free iodine is reduced to iodide.

An excess of unlabelled NaI or KI as a carrier for the high specific activity [^{125}I] iodide, and a protein described in buffer is added to act as a carrier for the labelled protein. The labelled protein is separated from the unreacted iodide, usually by gel filteration. The minimum concentration of oxidising agents needed to produce the required utilisation of isotope (and hence specific activity) should be employed. Higher concentrations are a potential cause of decomposition of the protein. The reaction appears to be instantaneous, and a minimum exposure of protein to potentially harmful reagents can be achieved by the addition of the reagents as rapidly as possible, followed immediately by the purificaiton of the labelled protein. Many workers claim that in some circumstances some delay is required after the addition of chloramine-T and before the addition of the reducing agent to enable high yields and high specific activities to be obtained. The chloramine-T reaction appears to have a pH optimum of 7.5, with the yields being reduced below pH 6.5 and above pH 8.5 (Ganguli and Hunter 1971; Freedlender 1969). High incorporation of radioiodine into proteins is generally obtained. Some proteins appear to be readily damaged by this method particularly by the formation of aggregated material (Sherman, Harwig and Hayne 1974), and the enzymic activity of some proteins seems to be lost after exposure to oxidising agents (Fang, Cho and Meltzer 1975).

4.1.2 **Material**

Na ^{125}I: 3.7 GBq/ml

0.25 M sodium phosphate buffer, pH 7.5

0.5 mg/ml chloramine-T in 0.05 M sodium phosphate buffer, pH 7.5

1.2 mg/ml sodium metabisulphite in 0.05 M sodium phosphate buffer, pH 7.5

0.1 N KI solution in phosphate buffer, pH 7.5

Antigen to be iodinated, 0.2-0.5 mg/ml in 0.05 M phosphate buffer, pH 7.5

The chloramine-T, sodium metabisulphite and KI solutions were freshly prepared, and the reaction carried out on ice.

4.1.3 **Method:**

(i) Iodination

2 µl aliquot of Na ^{125}I solution was dispensed into a polystyrene tube using an Eppendorf micropipette with a disposable tip. Antigen containing a minimum of 50 µg protein in 50 µl of phosphate buffer was diluted with 20 µl of PBS and added to the tube, followed by 10 µl of 1% chloramine-T initiated the reaction. The tube was agitated for 10 min and the reaction was stopped by using 10 µl of 2.4% sodium metabisulphite.

(ii) Separation of Labelled Protein from Unreacted Iodine

This relatively simple biochemical fractionation requires the separation of small molecules, NaI, from a larger peptide or protein. A gel filteration method was used. A small (0.9 x 12 cm) column of Bio-Gel P-6 (60-100 mesh) (BioRad Laboratories, California, USA), presoaked in phosphate buffer, pH 7.5, was used for this purpose. 0.1 N KI and phosphate buffer solutions were used as eluant. The iodination reaction mixture was quantitatively

transferred to the prepared column and eluted with phosphate buffer. 1.0 ml fractions were collected into polystyrene tubes and their radioactivity as counted using a Packard gamma scintillation spectrometer. The column ran until both the protein and the free ^{125}I iodide peaks had been eluted and their radioactivity counted. Protein peaks were picked and their aliquots were stored at -70°C until required.

4.2 BIOSYNTHETIC LABELLING OF LARVAE

Parasite surface antigens can be labelled either by radioiodination or biosynthetically by incubation of larvae in culture media containing radioactive precursors. The latter procedure has the advantage over surface radioiodination in that the identified antigens are synthesized by the parasite. With radioiodination extraneous molecules strongly adsorbed to the parasite surface (Colli *et al.* 1981) may be labelled.

Parasite antigens can be labelled by the incorporation of [^{35}S-] methionine into their proteins (Sugane, Howell and Nicholas 1985). This thesis reports successful labelling of *T. canis*, *T. catis*, *T. pteropodis*, *A. suum* and *A. cantonensis*.

4.2.1 Material and Methods

The eggs from these parasites were hatched and cultured as described in Chapter 2. The hatched larvae were washed once in methionine-free Modified Eagles Medium (DMEM) (Flow Laboratories, UK) and then incubated for 6-12 h in fresh methionine-free medium to which was added 100 units/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml fungizone. To label, a thick suspension of over 100,000 larvae was cultured in 2 ml of this DMEM containing 54.6 T Bq/mol ^{35}[S-]methionine (Radiochemical Centre Amersham UK) in a Leighton tube. A stream of 5% CO_2 and 95% air was bubbled through the medium for 10 min

before closing the tube with a silicone stopper and then incubating at 37°C. The original DMEM was supplemented with 5 µl of radiolabelled [^{35}S-]methionine with a concentration of 54.6 RBq/ml and 10 µl of 2.92% glutamine and the pH was maintained at pH 7.4 by gassing in a fume cupboard (solution changed until cherry red).

10 µl of the labelled culture sample was taken and pipetted on to glass filter paper to estimate initial radioactivity of the culture solution. The cultures were incubated for 24-72 hr at 37°C and examined from time to time to check the viability of larvae and absence of contamination in the culture. After 24 hr larvae were allowed to settle, the supernatant was removed and the radioactivity of the culture sample measured. After 48 hr the culture medium was collected and replaced with 2 ml of fresh methionine-free medium containing 54.6 T Bq/ml [^{35}-S] methionine. Cultures were terminated between 7-21 days, depending on the viability of different parasites. Samples were taken to estimate radioactivity on a glass filter paper. The supernatant was removed, purified, and concentrated by dialysis against 30% Aquacide (polyethylenegluycol, Calbiochem, USA) and protein was estimated by the Bradford method. The aliquots were frozen at -20°C till required for the experiments. The larvae were washed in Dulbecco's phosphate buffered saline (pH 7.2) and sonicated in 0.5 ml of sterile water at 10 kilocycles for 30 min at 4°C. The sonicate was centrifuged at 400 g for 2 min and the supernatant retained (somatic extract) so as to distinguish the ES antigen from somatic antigens.

Minimum essential medium Eagle (modified) with Earl's salts without glutamine and methionine

Ingredients	mg/l
L-arginine HCl	126.4
L-cystine disodium salt	28.42
L-histidine HCl H_2O	41.90

L-isoleucine	52.50
L-leucine	52.50
L-lysine HCl	73.06
L-phenylalanine	33.02
L-threonine	47.64
L-tryptophan	10.20
L-tyrosine disodium salt	45.02
L-valine	46.90
D-Ca pantothenate	1.00
Choline chloride	1.00
Folic acid	1.00
i-inositol	2.00
Nicotinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	0.10
Thiamine HCl	1.00
$CaCl_2.2H_2O$	264.9
KCl	400.0
$Mg/SO_4.7H_2O$	200.0
NaCl	680.0
$NaHCO_3$	2000
$NaH_2PO_4.2H_2O$	158.3
D-glucose	1000
Phenol red sodium salt	17.00

4.2.2 Estimation of Incorporated [^{35}S-] Methionine into Protein

To determine the extent of incorporation of radioactivity with proteins, which may have been secreted or excreted, by the larvae during the culture period aliquots of concentrated culture media were precipitated on a 2.1 cm diameter glass fibre disc (Whatman GF/A) with an equal volume of 20% aqueous trichloroacetic acid (TCA) for 20 min at 4°C. The TCA precipitation was washed twice with 10% ice cold TCA

followed by water and dried using absolute ethanol. This radioactivity was counted in a Beckman scintillation counter using a toluene scintillant cocktail with 0.5% (w/v) butyl PBD as scintillant (2(4'-L-butyl phenyl)-5-(4'-biphenyl)-1,3,4-oxadiazole, scintillation grade, Koch-Light Laboratories). The percent incorporation of radioactivity was calculated as:

$$\frac{\text{Total TCA-precipitable radioactivity recovered}}{\text{Amount of radioactivity added to culture}} \times 100\%$$

4.3 AUTORADIOGRAPHY

Radio labelled antigen samples were subjected to gel electrophoresis as described in Chapter 3. The dried gels were exposed at -70°c to Fuji RX medical X-ray film sandwiched between Cronex intensifying screens (Dupont De Nemous, Wilmington USA). Autoradiographs were developed using Kodak developer and fixer as described by the manufacturer. Molecular weights of the labelled proteins were determined by comparing the radiation mobilities with those of unlabelled protein of known molecular weight on the stained gel as described in the legend to Figure 4.1.

On some occasions, especially when low levels of radioactivity were present in the samples loaded, the gels were subjected to fluorography (Bonner and Laskey 1974) by impregnating them with Enhance (New England Nuclear, Boston USA) for 1 hr following destaining. Impregnated gels were then soaked in water for 1 hr to precipitate the scintillant in the gel before they were dried. This procedure resulted in a reduction in time of exposure to the X-ray film of up to 200% when compared with untreated gels.

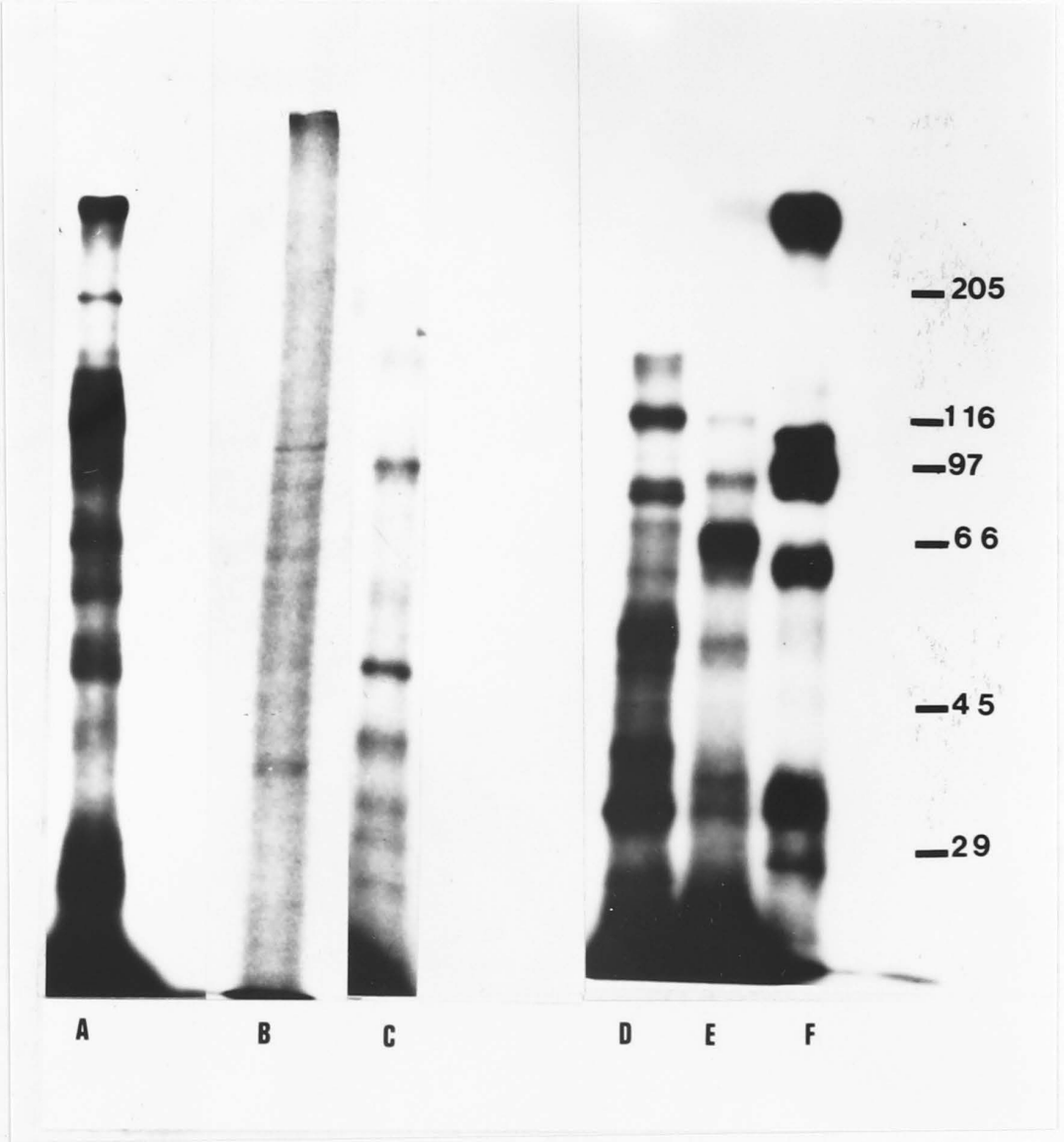


Fig. 4.1 Autoradiograph of SDS polyacrylamide gel (10%) showing the comparison of stage and species specific antigens.

Lane (A) surface labelled (^{125}I) adult *A. suum* ES antigen

Lane (B) biosynthetic labelled [^{35}-S] *A suum* larval ES antigen

Lane (C) surface labelled (^{125}I) adult *T. canis* ES antigen

Lane (D) as (C) but antigens from ligatured parasite

Lane (E) (^{125}I) labelled cuticle extract of *T. canis* adult

Lane (F) *T. canis* larval ES antigen surface labelled (^{125}I)

4.4 RESULTS

Antigens obtained from different nematodes were analysed by SDS-PAGE. They were characterised by three different methods:

1. Staining of polypeptide bands by Coomassie blue.
2. Autoradiography of surface labelled antigens using ^{125}I.
3. Autoradiography of biosynthetic labelled antigen using ^{35}S.

Each method is influenced by the biochemical nature of those antigens. Most of the gels analysed were replicated. *D. roemeri* and *T. leonina* were scarce and so could not be replicated with the same method twice, but enough material was available to analyse their antigens by two different methods.

The mean RF values with 95% confidence level of markers within gels (Fig. 4.2). The variation between lanes within the gels was negligible compared to the variation between the gels (Fig. 4.2 and Fig. 4.3). Standard molecular weight markers from two different suppliers were run on the same gel (Biorad and Sigma) to assess the variations.

4.4.1 Analysis of Different Molecular Weight Peptide Bands Obtained from Relevant Nematode Antigens

T. canis

Both adult *T. canis* ES antigens and *T. canis* larval ES antigens gave similar peptide bands between 90-116 KD (Fig. 4.1, Lane C Lane F), but the larval ES antigens also had two prominent high molecular weight bands between 168-198 which were specific to *T. canis* larvae. Adult cuticular extracts had very few peptide bands and none over 116 KD (Fig. 4.1, Lane E).

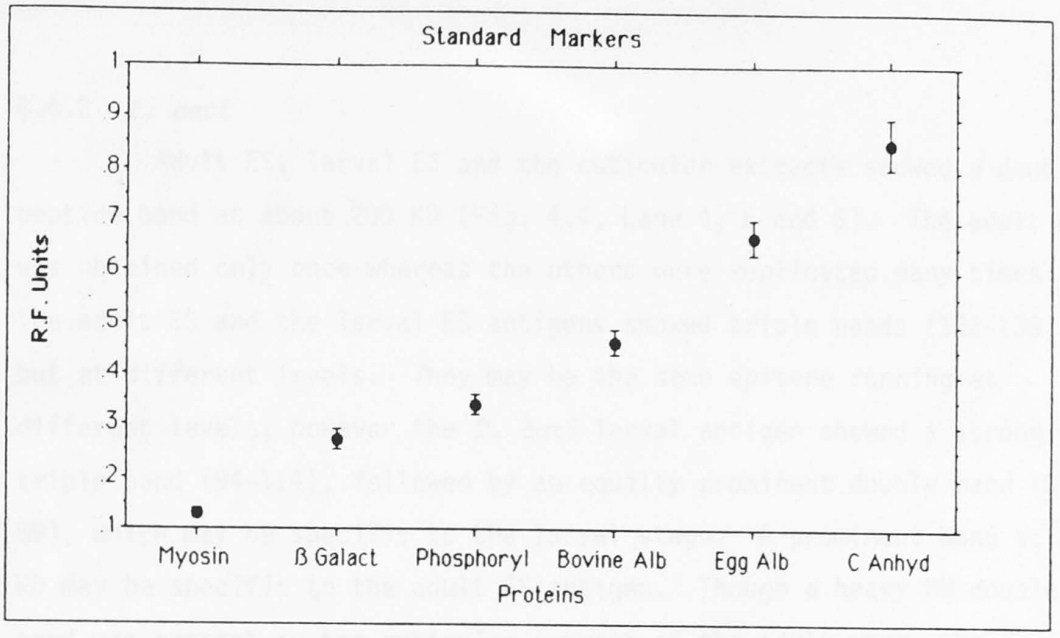


Fig. 4.2 The mean RF values with 95% confidence level of markers within gels

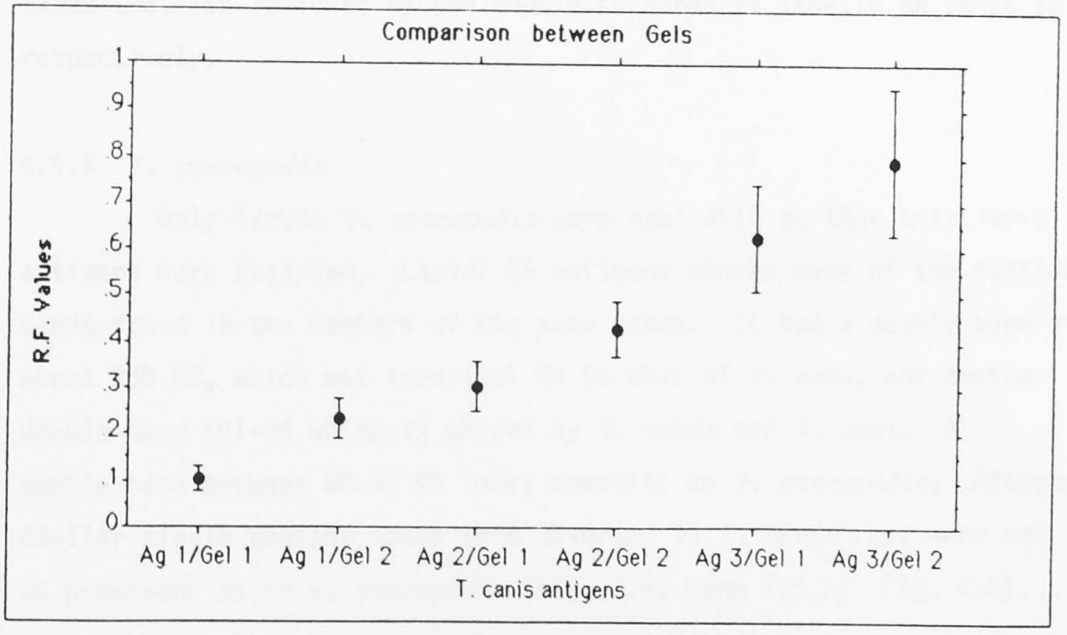


Fig. 4.3 The mean RF values with 95% confidence level of antigens between gels

See Appendix III

4.4.2 *T. cati*

Adult ES, larval ES and the cuticular extracts showed a double peptide band at about 200 KD (Fig. 4.4, Lane 4, 5 and 6). The adult ES was obtained only once whereas the others were replicated many times. The adult ES and the larval ES antigens showed triple bands (122-138), but at different levels. They may be the same epitope running at different levels, however the *T. cati* larval antigen showed a strong triple band (94-114), followed by an equally prominent double band (87-89), which may be specific to the larval stage. A prominent band at 38 KD may be specific to the adult ES antigen. Though a heavy MW double band was present in the cuticular extract of the adult worm, the antigenic peptides (see Chapter 5) from 94-138 KD were clearly absent on the cuticular extract. The *T. cati* antigens obtained biosynthetically always exhibited a double band at 200 followed by a triple band (122-138) and a double band at 87-106, whereas the *T. canis* had two single bands 198, 168 followed by two double KD bands at 128-116 an 98-96 KD respectively.

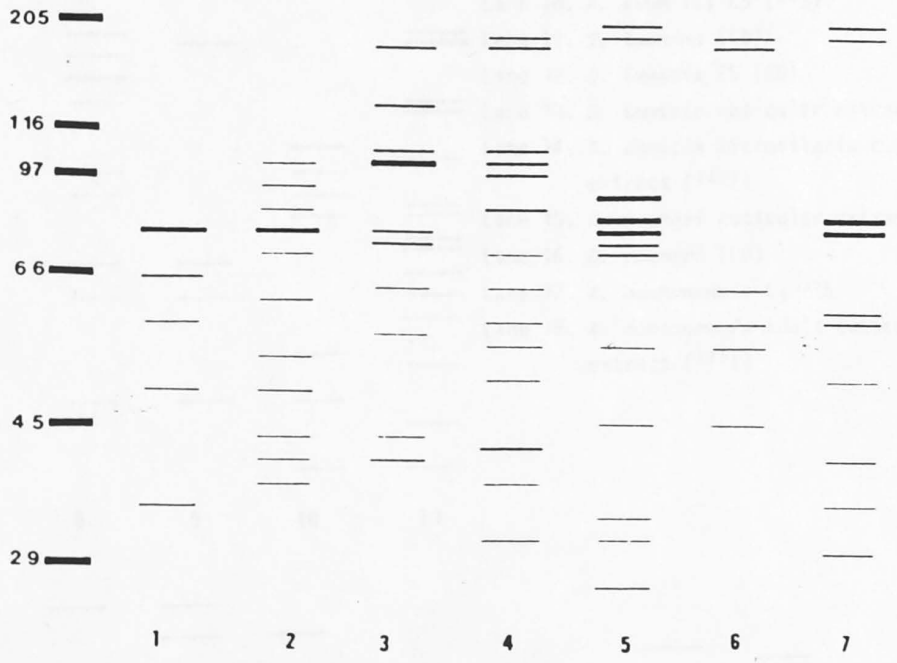
4.4.3 *T. pteropodis*

Only larval *T. pteropodis* were available so that only larval antigens were analysed. Larval ES antigens showed many of the peptide bands found in the members of the same genus. It had a double band of about 200 KD, which was identical MW to that of *T. cati*, and another double band 101-96 which is shared by *T. canis* and *T. cati*. A double band between 68-56 KD looks specific to *T. pteropodis*. Although similar single peptide bands were observed in *T. canis* they were not as prominent as in *T. pteropodis* (Fig. 4.4, Lane 3,5,7; Fig. 4.6).

4.4.4 *T. leonina*

Sufficient *T. leonina* was available to make only two stained gels. It shared all the major peptide bands found with *T. canis*

Fig. 4.4 Schematic representation of peptide bands from antigens obtained from different parasites.

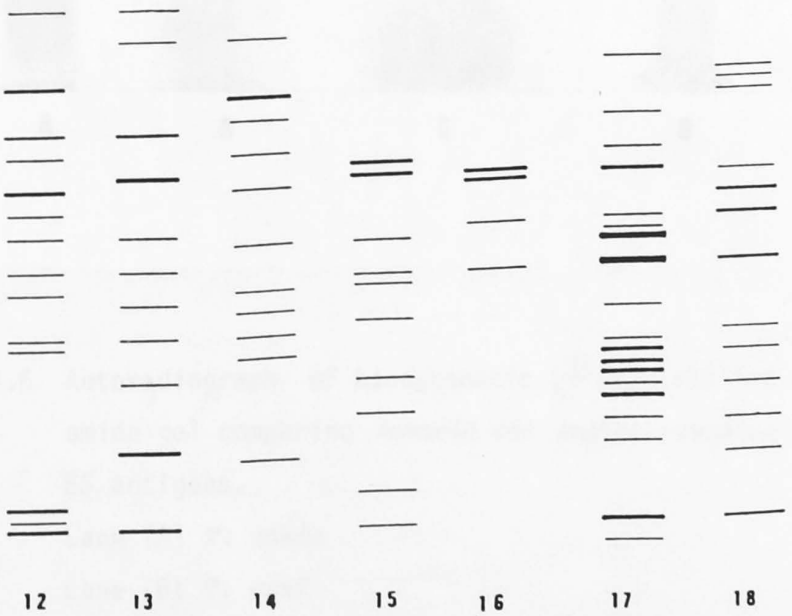


Lane 1. *T. canis* adult cuticular extract (^{125}I)
Lane 2. *T. canis* adult ES (^{125}I)
Lane 3. *T. canis* second stage larval (L) ES (^{125}I)
Lane 4. *T. cati* adult (ES) (^{125}I)
Lane 5. *T. cati* L ES (^{35}S)
Lane 6. *T. cati* adult cuticular extract (^{125}I)
Lane 7. *T. pteropodis* (L) ES (^{35}S)

Fig. 4.5

Schematic representation of peptide bands from antigens obtained from different parasites.

Lane 8. *A. suum* adult ES (^{125}I)
Lane 9. *A. suum* cuticular extract (^{125}I)
Lane 10. *A. suum* (L) ES (^{35}S)
Lane 11. *T. leonina* (CB)
Lane 12. *D. immitis* ES (CB)
Lane 13. *D. immitis* cuticular extract (CB)
Lane 14. *D. immitis* microfilaria cuticular extract (^{125}I)
Lane 15. *D. roemeri* cuticular extract (CB)
Lane 16. *D. roemeri* (CB)
Lane 17. *A. cantonensis* L_3 ^{35}S
Lane 18. *A. cantonensis* adult cuticular extract (^{125}I)



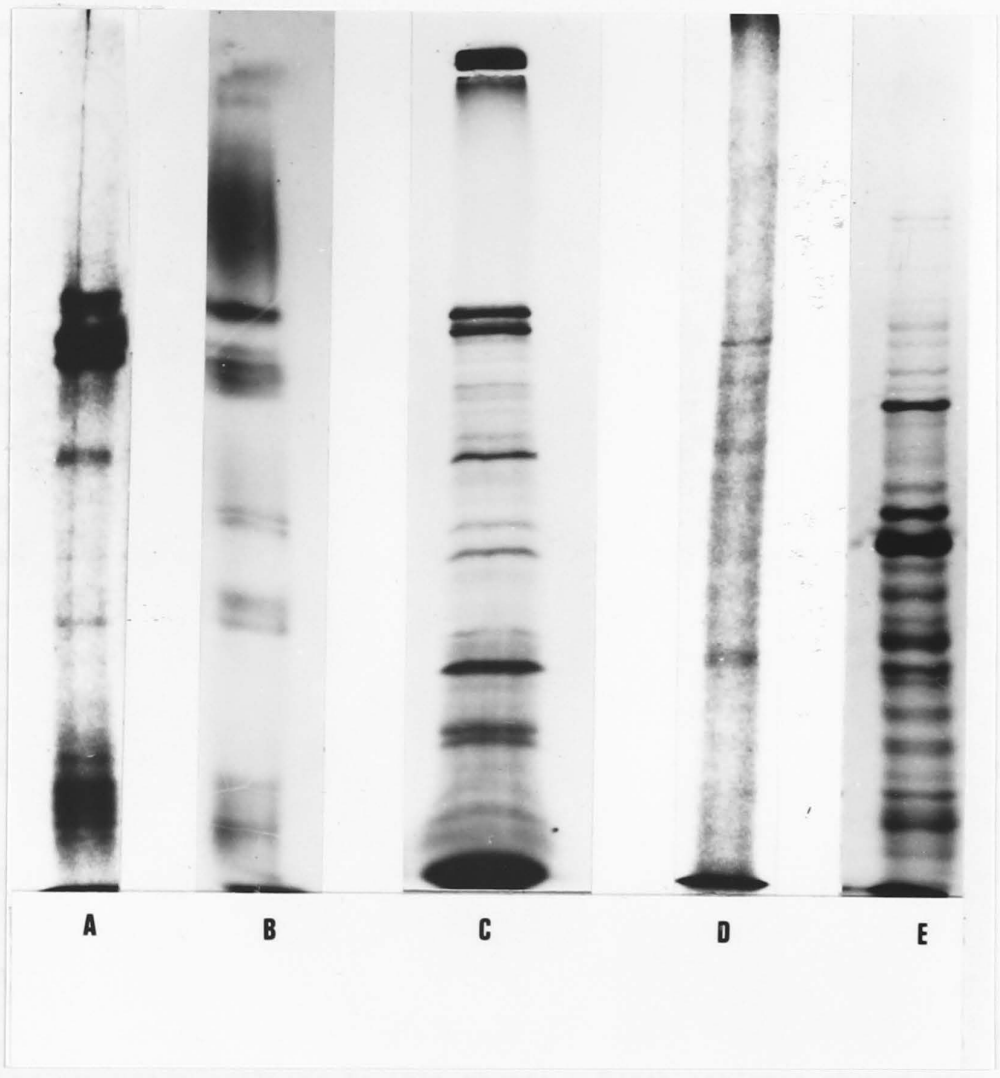


Fig. 4.6 Autoradiograph of biosynthetic [^{35}S-] labelled SDS polyacrylamide gel comparing *Ascarid* and *Angiostrongylus* larval ES antigens.

Lane (A) *T. canis*

Lane (B) *T. cati*

Lane (C) *T. pteropodis*

Lane (D) *A. suum*

Lane (E) *A. cantonensis*

adult ES antigens, except for three double bands between 75-65, 62-56 and 55-47 KD, which were characteristic of *T. leonina* on silver staining (Fig. 4.5, Lane 11).

4.4.5 *A. suum*

A. suum shared four peptides with MW of 213, 77-67, 51-43 and 27 KD, common to larvae and adults (Fig. 4.5, Lane 8-10). Also extracted from the cuticle were peptides with MW of 213, 125, 51-43, 27 KD. The adult ES antigen had a single peptide at 164, followed by a triple at 138-126, and a double band between 111-103 KD. The triple band seems to be specific to the adult (Fig. 4.1, Lane A-B). A peptide at about 83 KD appears to be specific to the larval stage of *A. suum*. When *A. suum* was compared to *T. canis* adult ES, the range of distribution of peptides was wide, from 213-27 KD in *A. suum*, whereas in *T. canis* the range was 160-44 KD (Fig. 4.1, Lane A and C). Furthermore, the prominent peptide is between 130-125 whereas in *T. canis* larval ES they are between 116-90 KD (Fig. 4.1, Lane F). Although a high MW peptide band is present at 213 in *A. suum* larval antigen, there is a characteristic absence of peptide bands 198-96 KD which are found in other ascarids.

4.4.6 *Dirofilaria immitis*

Filarial antigens were unstable compared to the antigens from other parasites analysed in this thesis. Fresh antigens showed more peptides stained with Coomassie blue than the antigens radiolabelled the following day. Adult ES antigen showed peptide bands ranging from 21-185 KD (Fig. 4.5, Lane 12). The characteristic bands were at 138, (116-114), 91 and (23-21) KD. The 138 KD and a triple band at 23-21 KD seems to be specific to ES antigen. Although the cuticular extract had a similar 21-29 KD band, the pattern differs and 138 KD was absent in the cuticular extract (Fig. 4.5, Lane 13). The peptides around 138-128 KD are shared by the stages of *D. immitis* (Lane 12-14). High MW antigens 185-194 KD were absent in the (L35) microfilaria (Lane 25).

28 KD peptide may be specific to the microfilaria. Radioiodionated *D. immitis* adult ES antigens failed to show peptides other than 126, 106 and 41 KD. Detergent treated extract did not show any of the high MW peptide bands.

4.4.7 *D. roemeri*

D. roemeri antigens were also unstable like those of *D. immitis*. Adult ES antigens showed a double peptide band between (94-100) (Fig. 4.5), 72 and 63 KD whereas the detergent extracted cuticular antigen showed very low molecular weight peptide bands including 22-24 KD. Peptides between 21-34 KD may be specific to the Filaridae (Fig. 4.7) (Fig. 4.5, Lanes 12-16).

4.4.8 *Angiostrongylus cantonensis*

Biosynthetic labelled *A. cantonensis* larvae released antigens ranging from 23-203 KD. A double peptide between 94-96 KD (Fig. 4.5, Lane 17), a triplet between 60-61 and a single 54 KD were consistantly prominent bands. Surface labelled adult cuticular extract showed a triplet band between (189-160) KD, another triplet band between (96-116) KD and a single at 79 KD (Fig. 4.5, Lane 18). The polypeptide bands between 54-67 KD may be stage specific in *A. cantonensisis* third stage larvae. There were no characteristic differences in peptides of antigens cultured at 37°C and 20°C.

All these parasites expressed a consistent replicable peptide pattern of antigens. They shared common peptides around 200 KD and (94-98) KD. Species specific antigens were found in many of them. Ascarids expressed a bundle of peptides between 90-140 KD. *Toxocara* can be differentiated by the narrow distribution of those bundles between 90-110. A picture of one single and two double bands between 116-128 KD and a persistent 32 KD are characteristic peptide patterns of *T. canis*, whereas *T. cati* has a similar peptide bundle at a

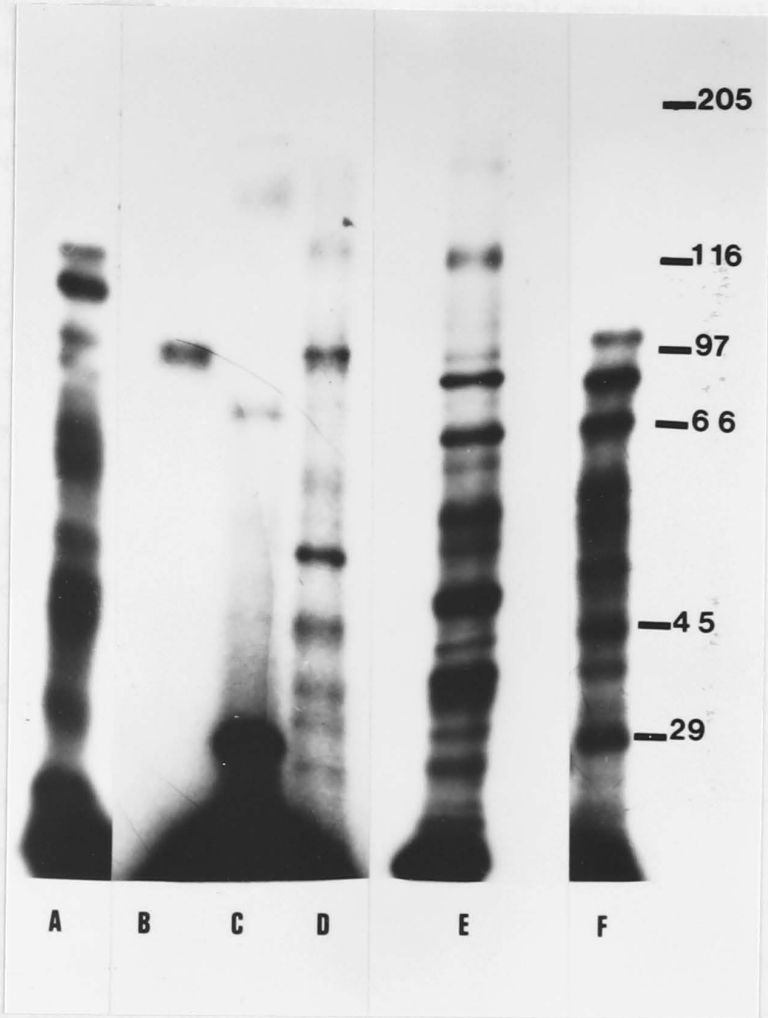


Fig. 4.7 Autoradiograph of filarial antigens. Comparison with ascarid surface labelled (^{125}I).

Lane (A) *D. roemeri* adult cuticular antigen

Lane (B) *D. roemeri* adult ES antigen

Lane (C) *D. immitis* adult ES antigen

Lane (D) *T. canis* adult ES antigen

Lane (E) cuticular extracted adult *D. immitis* antigen

Lane (F) *D. immitis* microfilarial antigen (cuticular extract)

lower level with a single followed by closely running double peptide bands between 94-110 KD. A double band between 56-68 KD is characteristic of *T. pteropodis*. Though *T. leonina* has peptide bands in the commonly shared region, the cleavage expressed is different from other ascarids. A double band between (119-130) KD and (96-111) KD, and another bundle of peptides between (65-75) KD, (56-62) KD are characteristic of *T. leonina* (Lane 11). Filarial parasites do show peptides between (94-98) KD but the peptide pattern is different from the ascarids. Furthermore, two closely running bands between (21-23) KD are characteristic of dirofilarial parasites. *D. roemeri* always show a double peptide band between (90-110) KD unlike *D. immitis*. *A.cantonensis* is characterised by a bundle of peptides between (59-67) and 54 KD.

4.5 DISCUSSION

It has been shown that a number of nematode parasites of mammals liberate antigens into culture media, or possess antigens which can be extracted by detergent from their cuticles (Maizels, Phillip and Ogilvie 1982). Nematodes analysed in this thesis share peptides of similar molecular weight. They may be stage or species specific. Little is known of their function, but they are clearly important in immunodiagnosis.

T. canis larval ES antigens constitute a series of glycoproteins, each of which have been shown to possess a distinct set of biochemical characteristics (Meghgi and Maizels 1986). In PAGE gels they are dissociated into peptides, with associated carbohydrates. Low molecular weight peptides between 32-70 KD are found to be a major allergen characterised in *T. canis* ES by Sugane and co-workers (Sugane and Oshima 1983). At 90-110 KD a triple peptide band which is prominent on [^{35}-S]methionine labelled ES antigen was proved to be antigenic (Sugane, Howell and Nicholas 1985). 120 KD peptide is a triple closely migrating glycosylated peptide band (Meghi *et al.* 1986).

Coomassie blue failed to stain, but there is a rapid uptake of labelled methionine which is observed in the Boulton Hunter technique too. Higher molecular weight bands of 400 KD were not found on 10% non-gradient gel, however similar bands were observed on Western blot (Chapter 5). All the *T. canis* experiments were highly replicable.

The 90-99 KD peptide bands may be stage specific in *T. cati*. The absence of bands from 94-138 KD in cuticular extracts is an indication that these are mainly excreted or secreted. The bands at about 200 and 58 KD were observed in adult and larval ES and cuticular extracts. A triple band between 122-138 KD is probably specific to adults. Although similar peptides are found in *T. canis* antigens, the pattern in which they are arranged is different, probably due to the biochemical structure of the glycoprotein. Unlike the *T. canis* larval ES, the *T. cati* larval ES has a triple band between 94-111 followed by a double band between (87-90) KD. Genus *Toxocara* probably shares some polypeptides with molecular weights between 99-110 KD.

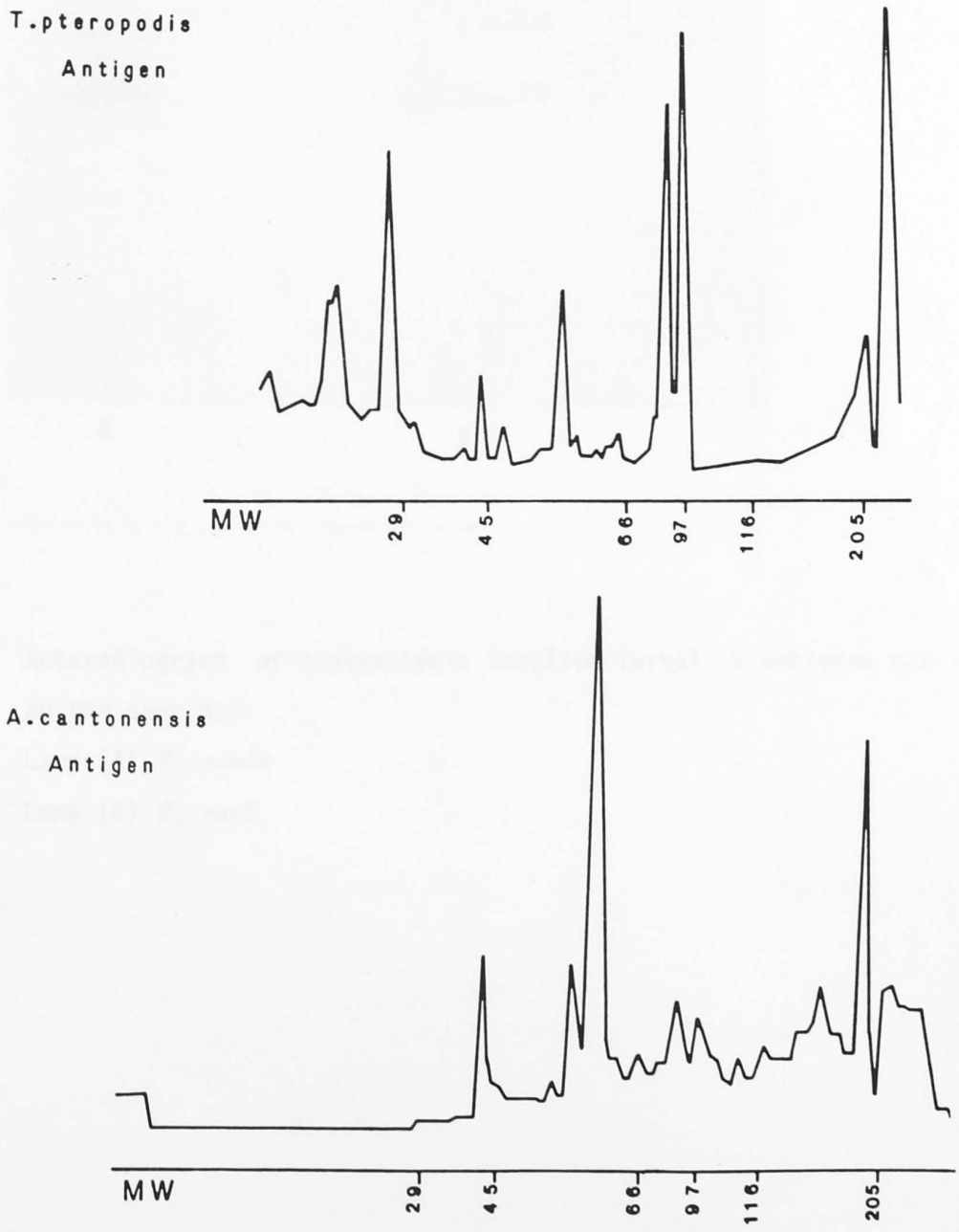
Fresh antigens stained with Coomassie blue showed the peptide bands at 185, 138, (91-114) (85-46) (21-23) KD. The low molecular weight corresponds to the findings of Fujita and Tshidate (1984). Filarial antigens were found to be unstable probably due to the presence of Cathepsin D-like proteases (Sreedharaswamy and Jaffe 1983), however fresh antigen extracts at pH 8.2 did not show any loss of peptide bands. The loss of certains bands were found on ^{125}I surface labelled antigens. It may not be due to complete degradation because high molecular weight bands at 176, 106 KD and one low molecular weight band at 41 KD was found on the autoradiograph. This probably is due to the selective action of proteolytic enzyme which is found in the filarial cuticle.

Surface labelled cuticular extract of *D. immitis* showed a striking absence of high molecular weight bands and an increased number

of double peptide bands. This may be due to the preferential labelling of the low molecular weight proteins by the Chloramin T method, as observed by Kaushal, Hussain, and Ottesen (1984). The presence of high molecular weight bands on the ES antigen of adult *D. immitis* suggest that it is likely that the cuticular extract has been broken down. The antigens extracted from the microfilaria confirms the findings of Boto and coworkers (Boto, Powers and Levy 1984). Microfilarial antigens shared two peptides (176 and 106 KD) with the adult ES and two (44 and 49 KD) with adult cuticular extracts. The 28 KD peptide resembles a stage specific antigen proved to be antigenic in by Boto *et al.* (1984). Unlike *D. immitis*, the cuticular extracts of *D. roemeri* exhibited high molecular weight bands between 138-90 KD but the adult ES surface labelled antigens showed only one peptide band at 116 KD, probably due to insufficient ES material.

Filarial parasites shared peptides of similar molecular weight with ascarids between 116-128 KD, which may be interfering with their cross reactions (Fig. 4.7). Though *A. cantonensis* also shares the peptide bands between 116-128 KD, it differs in the pattern of distribution of peptides between 203-27 KD from other parasites (Fig. 4.8). In this they correspond to the findings of Dharmkrong-at and Slrisinha (1983). The bands between 54-67 KD are typical of *A. cantonensis* third stage larvae. Lower molecular weight helminth antigens may show greater species specificity than those with higher molecular weight which show greater cross-reactivity (Kaushal, Hussain and Ottesen 1984). At this stage of analysis it is difficult to differentiate specific low molecular weight peptides from the degraded peptide bands. Although there are many peptides of similar and specific molecular weight, one which are used as tools for immunodiagnosis depend on which peptides are being recognised by the host. An attempt is made to investigate this by immunoprecipitation and Western blot in the following Chapter.

Fig. 4.8 Scanning of various peptide bands as shown by microdensitometer



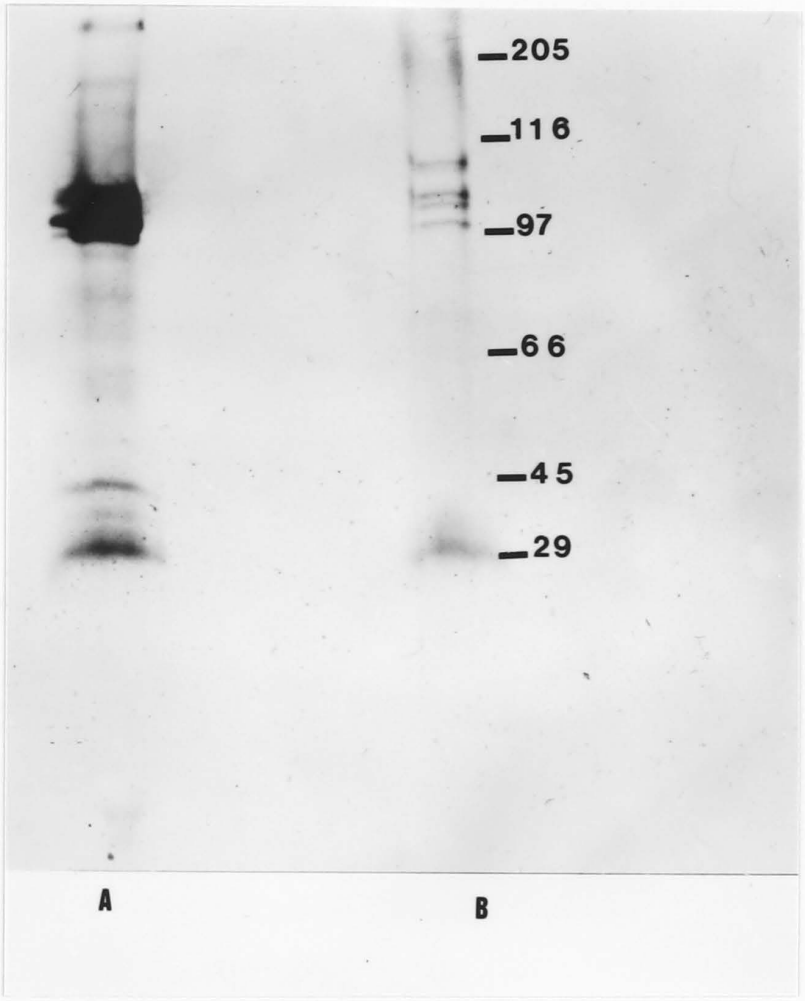


Fig. 4.9 Autoradiograph of biosynthetic labelled larval ES antigens run on the same gel.

Lane (A) *T. canis*

Lane (B) *T. cati*

Table 4.2 The incorporation of radioactivity labelled amino acids into infective stage parasites.

Antigen	Protein µg/ml	Radioactivity* of culture (CPM)	Radioactivity of* TCA precipitable material (CPM)	Incorp-oration (%)
T. canis	1.92	3251500	68950	2.12
T. cati	8.8	41811100	63600	1.53
A. cantonensis	7.42	3027600	345750	11.42
A. suum	3.01	2631800	74900	2.8

*From 10 µl of medium

Total volume of medium for each incubation was 2 ml.

As the radioactive counts were taken over a glass filter paper it is difficult to calculate the counting efficiency. Therefore the radioactivity was expressed in counts per minute (CPM) and not degradation per minute (DPM).

CHAPTER 5

CHARACTERIZATION OF THE EXCRETORY-SECRETORY ANTIGENS OF NEMATODE PARASITES

5.1 INTRODUCTION

The excretory-secretory antigens of nematode parasites have been shown to provoke a protective immune response in mice to *T. canis* (Nicholas, Stewart and Mitchell 1984). Young and Dobson (1982) have also demonstrated this with *A. cantonensis*. Despite these observations, little is known of the composition of the ES antigens of these nematodes. The number of antigens present, their biochemical characterization and the relationship of antigenic structure to the widespread cross reactivity (Ogilvie and de Savigny 1982) have not been elucidated. Indeed, although it is recognized that the ES antigens of a number of helminth parasites evoke protective immune responses in various hosts, few have been isolated or characterized (Clegg and Smith 1978; Lloyd 1981).

An effective approach to the characterization of a parasite's antigens is to label them radioisotopically and then carry out immunoprecipitation experiments, followed by electrophoresis and autoradiography. These techniques have been applied to protozoan parasites, such as some of the species of *Plasmodium* (Brown *et al.* 1981; Myler *et al.* 1982) and *Babesia* (Mitchell *et al.* 1982), but rarely with parasitic helminths.

5.2. IMMUNOPRECIPITATION

This Chapter discusses the immunoprecipitation of radiolabelled antigens in order to observe which peptide bands were antigenic and an attempt was made to analyse the peptide bands which showed cross reactions.

5.2.1 Materials and Methods

T. canis, *T. cati* and *A. cantonensis* labelled antigens were obtained biosynthetically and *T. canis*, *A. cantonensis* and *D. immitis* adult antigens were obtained by radioiodination. The antigens were incubated for 18 hr at 4°C with equal volumes of the appropriate sera from either uninfected or infected mice (NMS or IMS respectively). Surface labelled *D. immitis* microfilarial cuticular extracts were incubated with *D. immitis* infected dog sera and infection free dog sera. Biosynthetically labelled *T. cati* and *T. canis* +ve and infection free human sera (human sera from clinically positive cases were obtained from Dr J. Walker of the Institute of Tropical Medicine, Sydney) (and infection free dog sera from Dr M.D. Rickard, Faculty of Veterinary Science, Univeristy of Melbourne).

Immune complexes were adsorbed by formaldehyde-fixed *Straphylococcus aureus* Cowan 1 strain (SAC) following the method described by Kessler (1978). The *S. aureus* was provided by Dr M.J. Howell, Department of Zoology, ANU. It was grown in a rich medium, consisting of 0.8% nutrient broth (Onoid), 1.0% casamino acid (Difco), 0.25% yeast extract (oxoid), 0.5% sodium β-glycerophosphate and the vitamins nicotinamide (4 μg/ml) and thiamine (2 mg/ml). Stocks were maintained at 4 °C on 0.6% agar slopes. Nonidet P-40 (NP-40, Sigma) was used as the nonionic detergent at a concentration of 0.05% (v/v), and albumin (Sigma) was also always included in the absorption steps at concentrations of 1 mg/ml.

Following adsorption by SAC, the immune complexes were washed three times with 0.05% NP-40 in net buffer (150 mM-NaCl, 5 mM EDTA, 50 mM Tris HCl, 0.02% N_3, pH 7.4). SAC immune complexes were processed directly for electrophoresis by boiling for 5 min in the sample buffer The bacterial adsorbent was then sedimented by centrifugation for 60 sec in an Eppendorf centrifuge and the supernatant was loaded onto a gel.

Electrophoresis was carried out as described in Chapter 3. The dried gel was exposed to an X-ray film for autoradiography following the method in Chapter 4.

5.2.2 Results

Autoradiographs of gels showed clearly that immune serum bound some antigens with each of the parasite extracts studied. Control normal serum were included with each gel. Coomassie blue staining prior to autoradiography revealed many peptides, but since most did not give autoradiographs, they were considered products of *Staphylococcus aureus*.

Mice immune to *T. canis* recognised a triple band between 110-120 KD (Fig. 5.1). With *T. cati* a triple band at 110 KD, a double band at 68 and 50 KD and a diffuse band at 20 KD proved to be recognised as antigens (Fig. 5.2, 5.3). With *A. cantonensis* larval ES antigens, three bands, one each at 86 KD, 60 KD and 42 KD were precipitated (Fig. 5.6), while with *A. cantonensis* surface labelled cuticullar extracts antibody precipitated more peptides at 186, 169, 125, 114, 104, 52 and 45 KD. Figure 5.3, Lane B and D, showing the immunoprecipitates with normal human and mouse sera respectively lack any bands labelled antigenic bands. Lane C and D, immunoprecipitates with sera from human *T. canis* and mouse *T. cati* infection respectively show patterns of bands similar to unprecipitated antigens.

Rat infected with *A. cantonensis* recognised three sets of bands (Fig. 5.5), namely two triple peptide bands between 125-186 KD and 84-114 KD. The latter resembles the 99-110 KD which was found in most of these nematodes. A set of double bands was found between 30-37 KD which were confirmed by autoradiography. Coomassie blue stained gel showed evidence of probable cross reaction of *T. canis* antigen to *A. cantonensis* (Fig. 5.4) but the autoradiography failed to show any

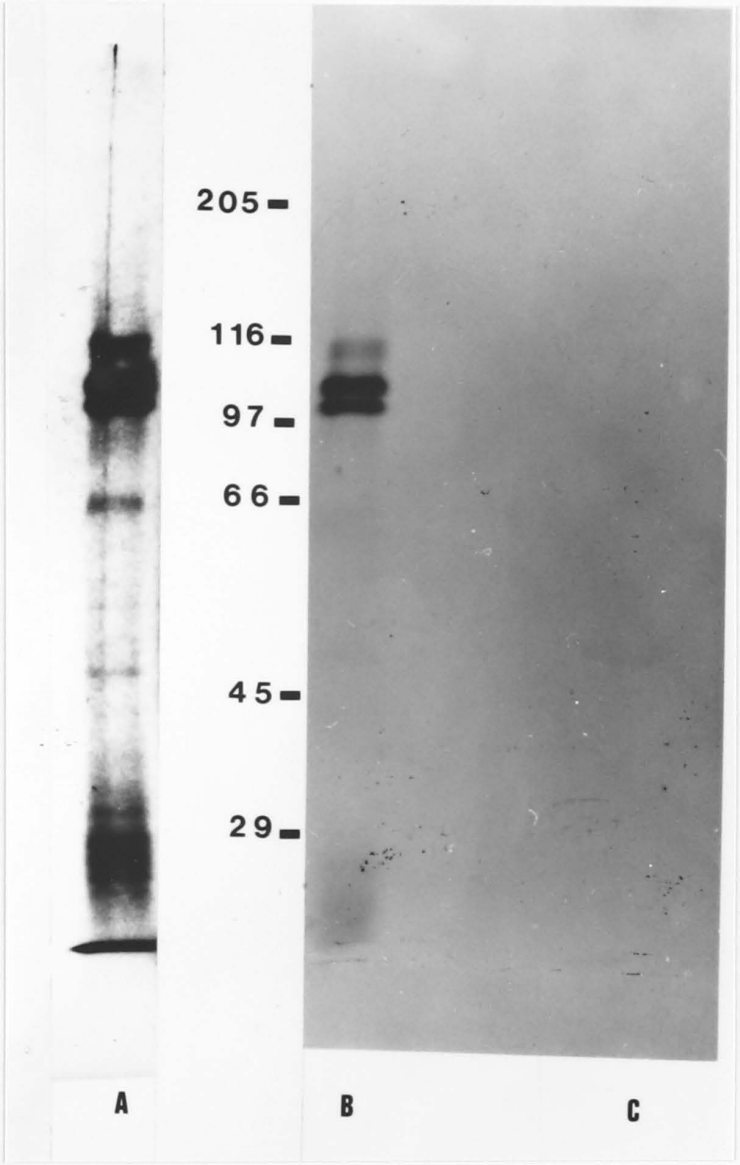


Fig. 5.1 Fluorograph of immunoprecipitation of *T. canis* antigen.
Lane (A) biosynthetic [^{35}S] labelled *T. canis* larval ES antigen
Lane (B) immunoprecipitates following incubation of ES antigen with *T. canis* infected mice sera
Lane (C) as for Lane (B) but negative mice sera

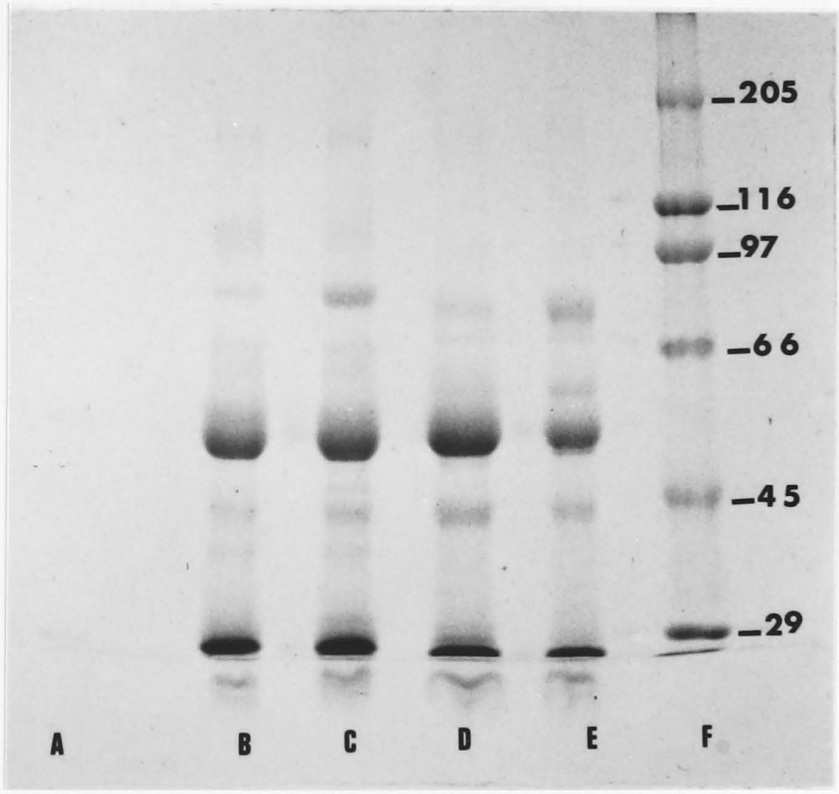


Fig. 5.2 Coomassie blue stained SDS-PAGE gel.

Lane (A) plain *T. cati* Ag with no serum

Lane (B) immunoprecipitate following incubation of antigen with infection free mouse serum

Lane (C) as for (B) but positive mouse serum

Lane (D) as for (B) but negative human serum

Lane (E) as for (C) but *T. canis* +ve human serum

Lane (F) MW markers

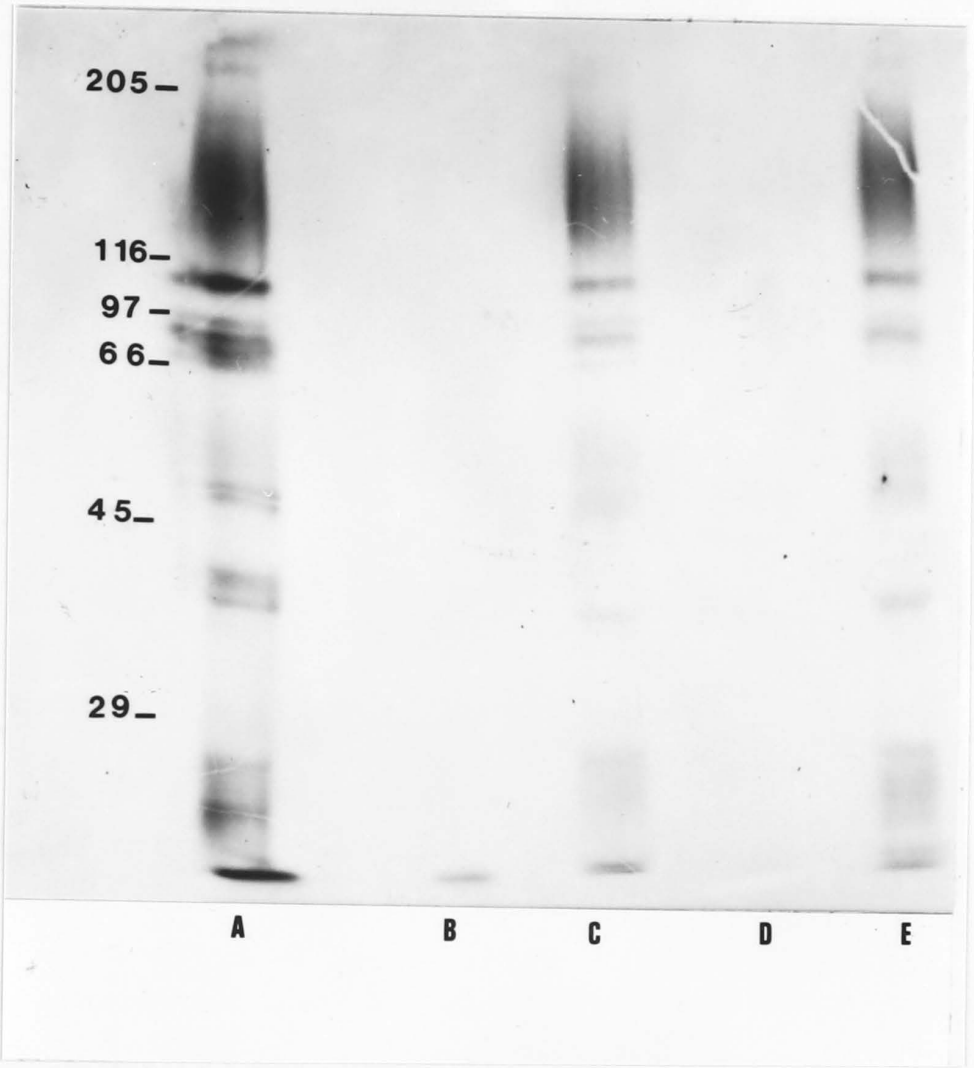


Fig. 5.3 Autoradiograph of the gel in Fig. 5.2.

Lane (A) *T. cati* ES antigen

Lane (B) immunoprecipitates following incubation of antigen with *T. cati* -ve mouse serum

Lane (C) as for (B) but +ve mouse serum

Lane (D) as for (B) but -ve human serum

Lane (E) as for (C) but *T. canis* +ve human serum

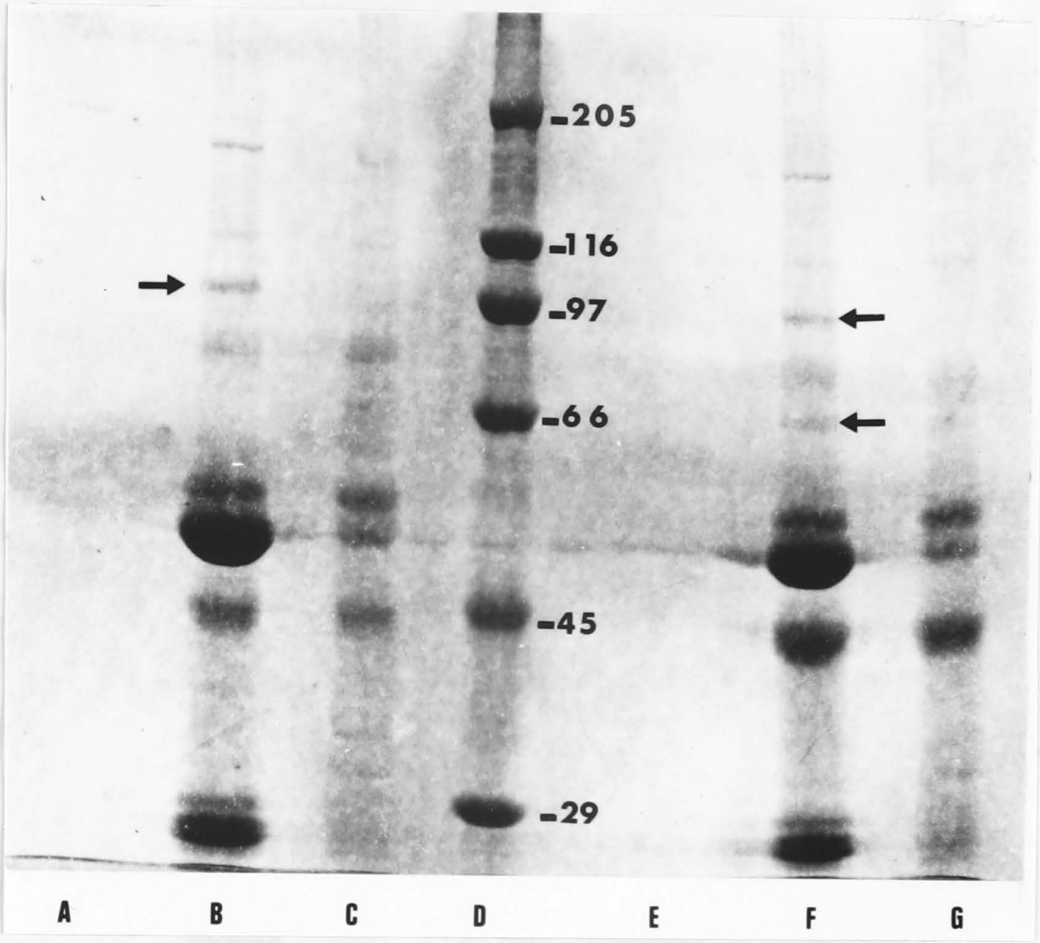


Fig. 5.4 Coomassie blue stained SDS-PAGE gel

Lane (A) plain surface labelled (^{125}I) adult *A. cantonensis* antigen

Lane (B) immunoprecipitates of *A. cantonensis* antigen with *Staphylococcus aureus* and *A. cantonensis* positive rat serum

Lane (C) as for (B) but normal rat serum

Lane (D) MW markers

Lane (E) [^{35}S] labelled *T. canis* antigen

Lane (F) as for (B) but *T. canis* antigen incubated with *A. cantonensis* antiserum

Lane (G) as for (F) but with normal rat serum

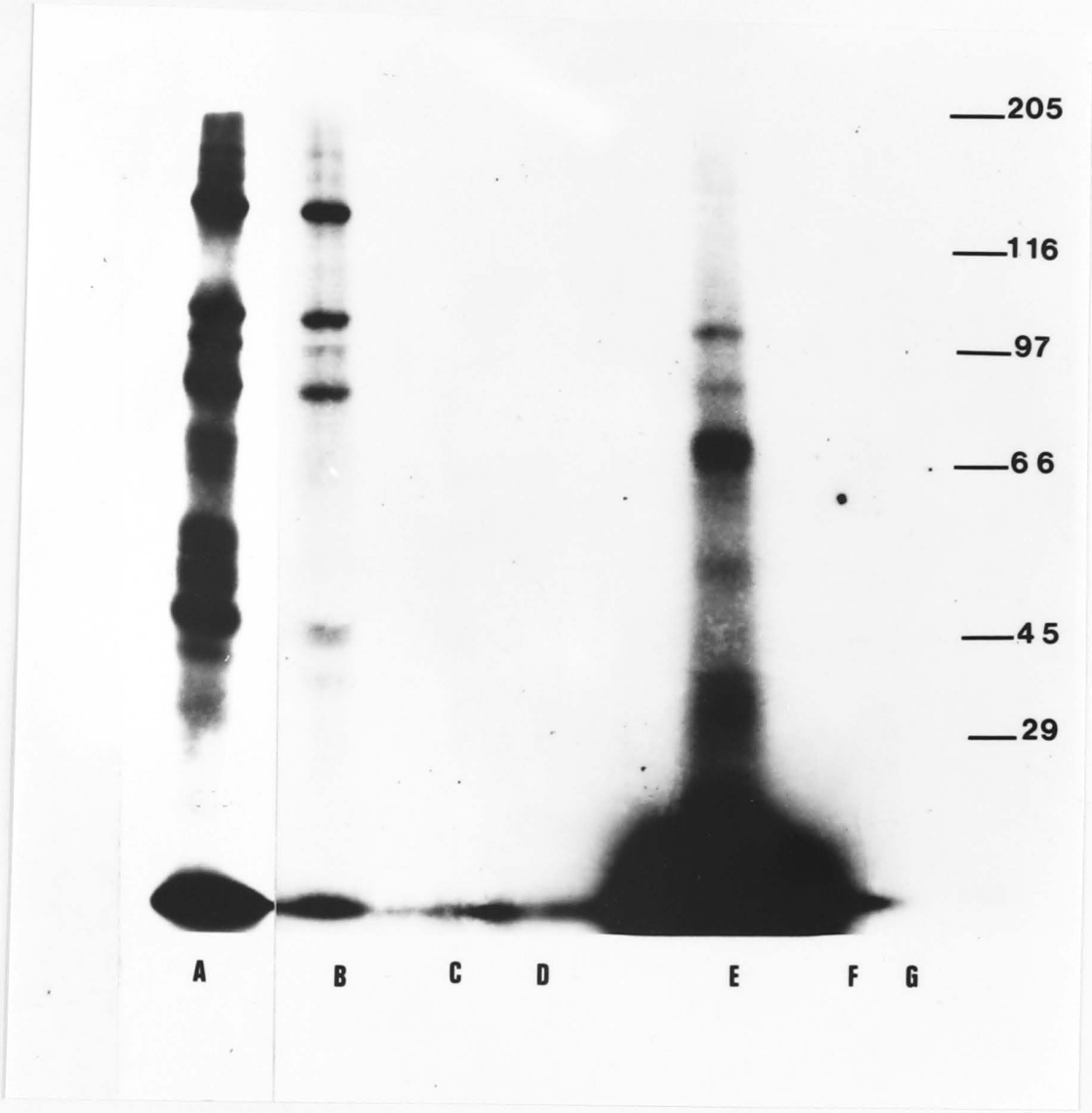


Fig. 5.5 Autoradiograph of Fig. 5.4

Lane (A) surface labelled (^{125}I) *A. cantonensis* cuticular antigen

Lane (B) immunoprecipitates of *A. cantonensis* cuticular antigens with same antiserum

Lane (C) as for (B) but with normal rat serum

Lane (D) MW markers

Lane (E) [^{35}S] labelled *T. canis* antigen

Lane (F), (G) no bands probably under exposed

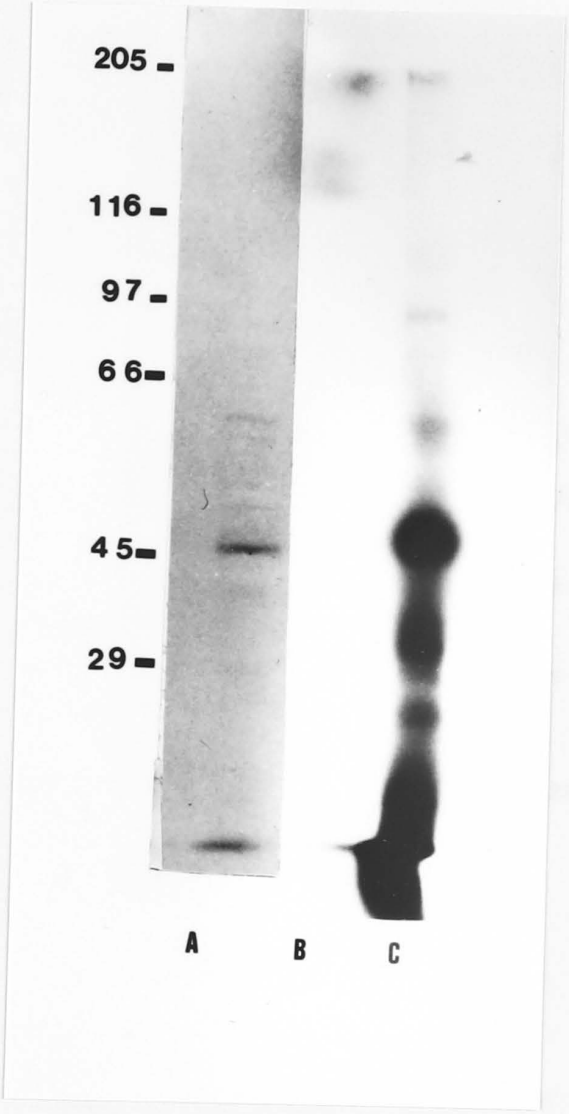


Fig. 5.6 Fluorograph of immunoprecipitation of *A. cantonensis* larval ES
Lane (A) antigen pretreated with *S. aureus* and incubated with *A. cantonensis* +ve rat serum
Lane (B) as for (A) but with infection free rat serum
Lane (C) plain ES antigen with no serum

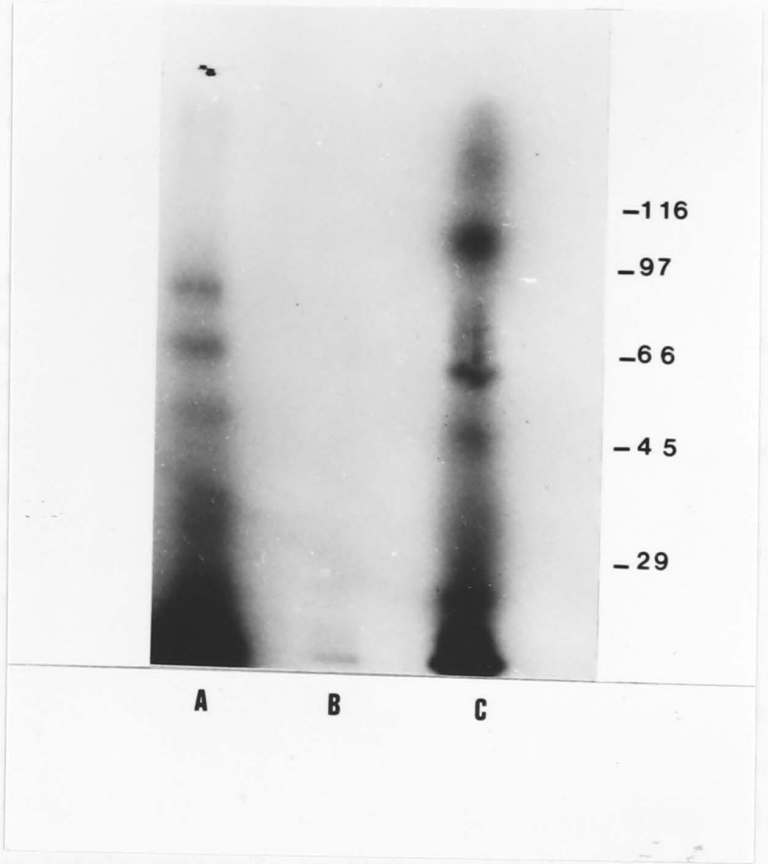


Fig. 5.7 Fluorograph of SDS-PAGE gel.

Lane (A) plain *D. immitis* microfilarial antigen (surface labelled ^{125}I)

Lane (B) immunoprecipates of infection free dog serum with microfilarial antigen

Lane (C) as for (B) but incubated with *D. immitis* +ve dog serum

bands on lane (F). This may be due to insufficient exposure time of the autoradiograph. Although lane (A) of Fig. 5.5 is from the same gel it was under exposed separately in order to show up the bands more clearly.

Sera from *D. immitis* infected dog precipitated *D. immitis* antigens at 112, 64, 48, 24, 20, 18 and 16 KD. Furthermore the same sera recognised a triple band between 110-120 KD with *T. canis* antigens.

5.2.3 Discussion

Immunoprecipitation shows which of the peptides bind serum IgG antibodies (since IgG antigen complexes are bound by protein A of *S. aureus*). Since only the antigens are labelled, autoradiography distinguishes antigen bound by serum IgG. Immunoprecipitation controls with normal serum shows that only traces of unbound or non-specifically bound antigens were presents on the gels.

The antigenic peptides recognised by the mouse and the human host parasitised by *T. canis* corresponded to the findings of Sugane, Howell and Nicholas (1985). *T. cati* peptides are probably homologous with the major triple antigenic peptides secreted by *T. canis* and variously reported to have molecular weight between 110-120 KD (Sugane, Howell and Nicholas 1985; Meghji and Maizels 1986). This triple band ran in the same position as the triple band from *T. canis* ES antigen.

Human sera from *T. canis* infection bind the antigens from *T. cati*, most significantly the triple peptide at 110-120 KD. One cannot know for certain whether or not the human clinical case of toxocariasis used for the positive serum had perhaps also been infected by other parasites in addition to *T. canis*, but with the mouse sera, it is certain that the antibodies were raised specifically against *T. cati*.

Since human antibodies to *T. canis* are bound by *T. cati* ES antigens, it is likely that antibodies raised by *T. cati* will bind to the principal *T. canis* ES antigens. Anti *T. cati* mouse serum reacts with *T. canis* ES antigens in Elisa tests (Nicholas, Stewart and Mitchell 1984).

A. cantonensis cuticular extract showed seven peptides recognising the host whereas larval ES antigen showed only three. However, the larval ES antigen is more reliable because they are biosynthetically labelled ES antigens whereas one cannot rule out the presence of host protein in a cuticular extract. Moreover, the chloramine T technique is reported to interfere with the somatic antigens of the host (Meghji and Maizels 1986). It was observed that *A. cantonensis* antigens are not as stable as *T. canis*, and that these antigens needed pretreatment with *S. aureus* to reduce the nonspecific binding. High molecular weight peptide bands disappeared during storage.

D. immitis immune sera also showed the triple band 110-120 KD on incubation with *T. canis* antigen. This is indicative that the serum had antibodies to *T. canis* which is quite possible because all three positive dog sera were collected from the dog pound where *T. canis* infection is quite common.

T. pteropodis antigens were incubated with *T. pteropodis* immune mouse sera but no characteristic complexes were formed. The number of larva used may not have been sufficient to produce antibody or techniques used were not suitable for this particular complex. This demands a more detailed study of the antigen/antibody complexes of this parasite. Immunoprecipitates were not carried out with other parasites due to insufficient antigen and immune sera.

5.3 WESTERN BLOTTING: ELECTROPHORESIS TRANSFERS OF PROTEIN FROM SDS-GELS TO NITROCELLULOSE AND AUTORADIOGRAPHIC DETECTION WITH ANTIBODIES

5.3.1 Introduction

The identification of protein antigens contained in complex mixtures has usually been achieved by radiolabelling the antigen mixtures and the use of antibody to immunoprecipitate the antigen. Unbound material was washed away, the antigen-antibody complex dissociated by SDS and the antigen analysed by acrylamide gel elecrophoresis. Since only the antigen was radioactive, autoradiography of the gel was capable of identifying the antigens. This form of analysis has several drawbacks. First, it requires that the antigens which were bound by the serum antibody must have been radiolabelled at high specific activity. This is not always feasible or desirable. Secondly, it may occasionally generate antigenicity. Thirdly, if the antigen consists of two polypeptides, it is difficult to decide which of the two antigenic determinants is recognised mutually by the antibody.

These problems may be overcome by using Burnette's technique (1981, reviewed by Gershoni and Palade 1983). Electrophoretic transfer is usually performed by the Towbin (1979) technique. The antigen containing mixture is separated by SDS-polyacrylamide gel electrophoresis, and the proteins are transferred by electrophoresis in a direction perpendicular to the plane of the gel onto a membrane (usually nitrocellulose). This technique is valuable for the analysis of proteins fractioned on the basis of molecular weight in SDS gel electrophoresis. The major limitation of the Western Blotting technique is that with low antigen concentration or weakly reactive antibody the specific rective bands may be difficult to distinguish from the background reactivity of either primary or secondary reagents. To prevent nonspecific binding Towbin (1979) used bovine serum albumin (BSA) or gelatin, both of which have been found to be relatively inefficient for the purpose (Johnson, Gutsch, Sportsman and Elder 1984). Thus, non-fat dry milk was used in the Westen Blot experiments.

The technique is useful for comparing the reactivity of several different sera with a single antigenic preparation (Hanff 1982) or comparing a single antibody preparation with several antigen source (Pekkala-Flagan and Ruoslahti 1982).

The Western Blot technique has three steps. First the components of an antigenic mixture (AGS) are separated by polyacrylamide gel electrophoresis (PAGE) in slab gels. The separated antigens are transferred electrophoretically, while maintaining their spatial relationships, to a sheet of nitrocellulose paper (NCP). Finally, the NCP is treated with an appropriate antibody solution or antiserum (AB), and the presence of the bound AB is detected by an enzymatic antibody probe. On addition of an enzyme substrate to an enzyme conjugated second antibody, colour is developed over the host's recognised bands.

5.3.2 Materials and Methods

Biosynthetically labelled antigens from *T. canis*, *T. cati*, and *A. cantonensis* were obtained as described in Chapter 4. Laboratory mice infected with one of these parasites and uninfected (control) mice were bled and sera collected as described in Chapter 2. The following materials were obtained from BioRad Laboratories, Hornsby, NSW: Affinity purified goat anti-mouse IgG - Horseradish peroxidase conjugate second antibody (GAM-HRP), Tween-20; ETA grade, pure nitrocellulose membrane, 9.2 x 15 cm; HRP colour development reagent containing 4-chloro-1-napthol, hydroxymethyl aminomethane (TRTS). A Biorad Transblot electrophoresis cell was used for the transfers.

Radiolabelled antigens from the above mentioned parasites were loaded onto six SDS polyacrylamide gels. Each gel carried samples of antigens from three parasites. *A. cantonensis* antigens were in two lanes, one harvested at 20°C incubation and the other at 37°C. They were subjected to electrophoresis under the same conditions as described

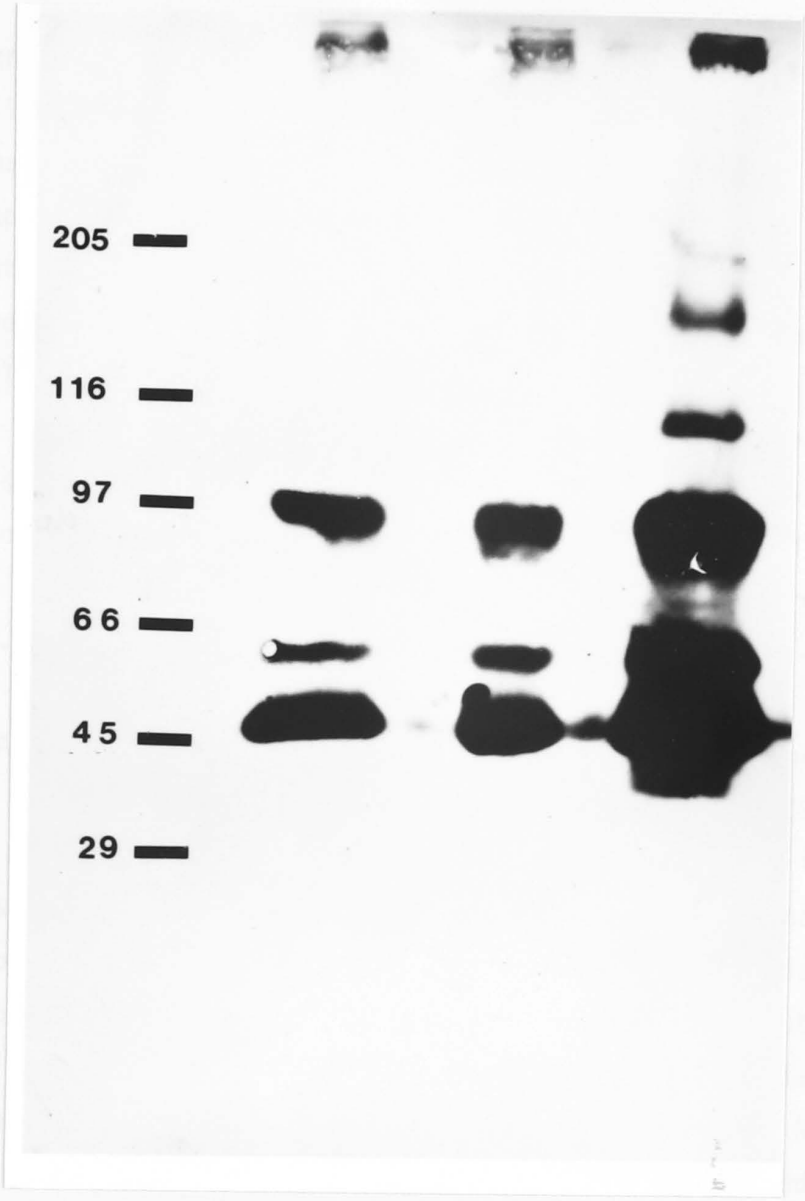


Fig. 5.8 Western Blot of *T. canis* larval ES antigen incubated with *T. canis* +ve mouse serum. Same antigen prepared on different days run on the same gel.

in Chapter 3. When the run was finished, the gel assembly was removed. The lane loaded with molecular weight markers was cut out and stained with Coomassie blue R 250 (Sigma). The gels were transferred onto a filter paper soaked in transfer buffer TB (20 mM Tris, 150 mM glycine in 20% methanol, pH 7.3). After soaking in TB the dull side of the NCP was placed in contact with the gel and covered by another layer of TB soaked filter paper. The gel and NCP along with the filter paper were placed in between two scouring pads (Scotch-brite, from the local hardware store) and was clamped with a plastic holder. This was slid into a BioRad Transblot system. The Transblot cell was filled with TB, placed on a magnetic stirrer and 150 mA current was passed through it overnight.

All the NCP were removed from the Transblot system and were immersed, at a 48° horizontal angle, in the locking solution (20 mM Tris, 500 mM NaCl pH 7.5, 5% 'Diploma' nonfat dry milk) (TBS) in a 20 x 20 cm glass dish. They were agitated on a rotary shaker for half an hour at room temperature. NCPs were transferrd to a second dish containing first antibody (either immune or normal serum) diluted in TBS/milk. Each NCP was incubated separately with the appropriate diluted serum solution for 1-2 hr with gentle agitation. The NCP were rinsed briefly with double distilled water and were transferred to a solution containing TBS/0.05% Tween 20 (TTBS) to remove free and non-specifically bound antibody. This was repeated three times with 10 minutes between the changes. Washed NCPs were incubated with the second antibody solution (goat anti-mouse TgG-horseradish peroxidase conjugate 1:3000 in TBS/milk) for an hour on a shaker platform. They were washed in TBS three times and a final two washes were given in TBS. These NCPs were treated with HRP colour development solution (BioRad) for 30 min. Colour development was stopped by immersing the NCP in distilled water for 10 min. NCP were air dried and preserved in a box.

5.3.3 Results

Most of the antigens used showed activity, but sera collected from mice infected with *A. cantonensis* failed to show any reaction. Sera from mice which had been infected with *T. canis* showed antibody binding with antigen from *T. canis* (Fig. 5.9) as well as from *A. cantonensis* (Fig. 5.10). Serum antibody from mice infected with *T. cati* bound to peptides from *T. canis* and *A. cantonensis* antigens. *T. canis* and *T. cati* antisera showed binding at the same level with *A. cantonensis*, but the *T. cati* sera showed weaker colour reaction. The data given in Table 5.1 lists the various peptides specific binding with different antibodies. The *T. cati* antigenic bands confirm the finding of the previous Chapter, which will be substantiated by Elisa in the following Chapter. Furthermore, *T. canis* and *T. cati* seem to be sharing the triple bands with molecular weights between 99-110 KD. The same sera were found to bind with an approximately 30 KD peptide of *A. cantonensis*. The latter bands had not been prominent in auto-radiogrpahs, but they were very prominent with the HRP conjugate reaction, as demonstrated in Figure 5.10. The control sera did not show binding on any of these reactive antigen lanes. Although the *A. cantonensis* larvae were incubated at two different temperatures and in separate lanes, the corresponding antigens did not show any differences in antibody or in peptide patterns.

5.3.4 Discussion

The limiting factors in this experiment were the low concentration antibody levels in the *T. cati* and *A. cantonensis* and quantifying the antigens. The difference in the intensity of the bands at the same level with *A. cantonensis* antigen incubated with *T. canis* and *T. cati* proves this. Thus, although *T. canis* infected mice sera showed binding with the corresponding *T. canis* antigen as well as with *T. catis* antigen, it did not exhibit a similar reciprocal binding which may be due to lack of optimum level of

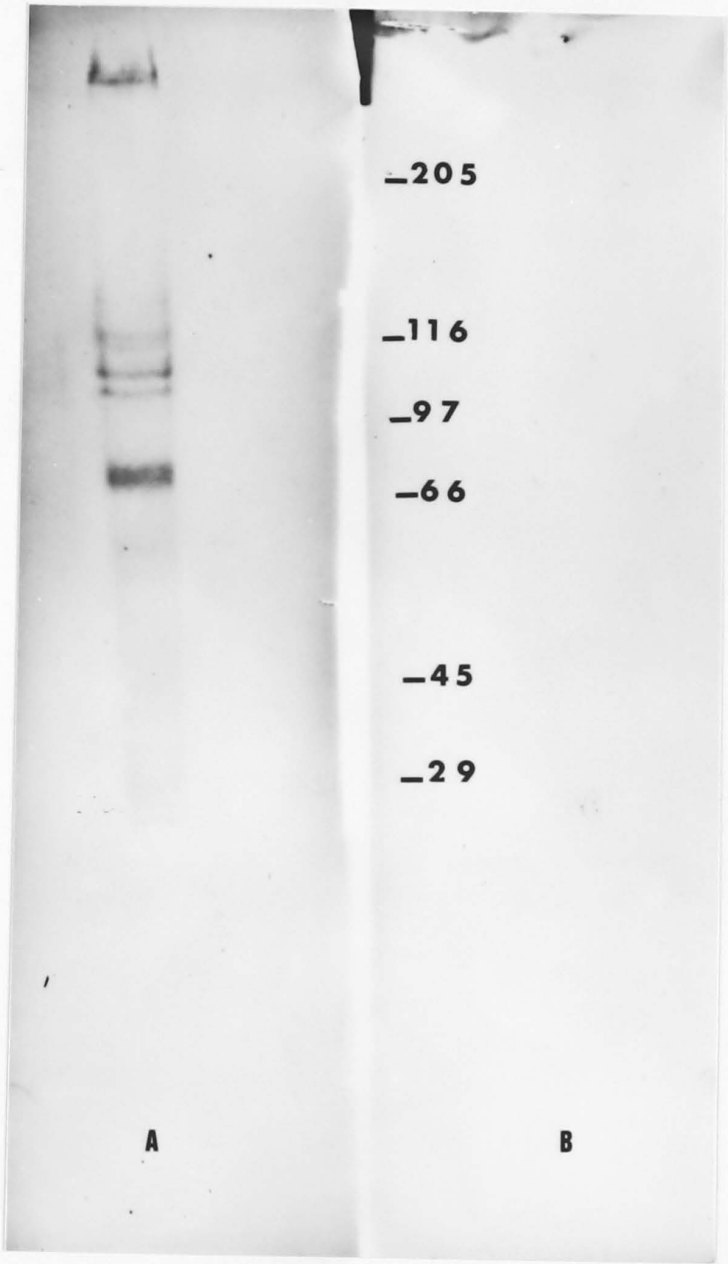


Fig. 5.9 Western Blot of *T. canis* larval ES antigen.

Lane (A) *T. canis* Ag incubated with *T. canis* +ve mouse serum

Lane (B) as for (A) but incubated with infection free serum

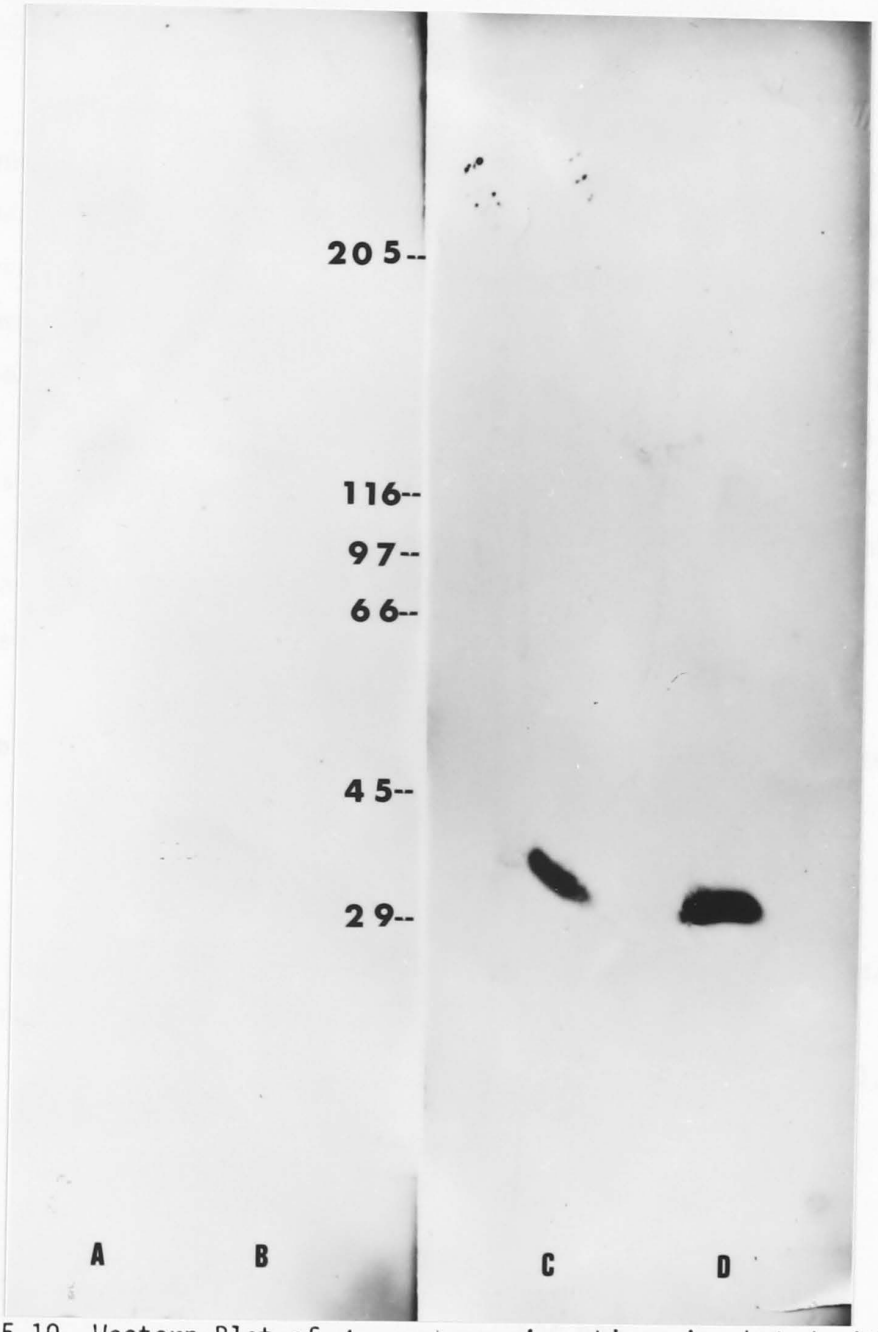


Fig. 5.10 Western Blot of *A. cantonensis* antigen incubated with *T. canis* mouse antiserum

Lane (A) *A. cantonensis* antigen cultured at 37°C incubated with normal mouse sera

Lane (B) as for (A) but with antigen cultured at 20°C

Lane (C) as for (A) but with *T. canis* antiserum

Lane (D) as for (B) but with *T. canis* antiserum

concentration of *A. cantonensis* antigen. It is essential to quantify the antigen in order to arrive at the optimal concentration level of AG and AB. Protein can be estimated but not the antigenic component of the protein. However, the colour reaction does show that there is a cross reaction between both *T. canis* and *T. cati* to *A. cantonensis*, the degree of which is highlighted by the Elisa tests. The antigenic band shown by *A. cantonensis* at approximately 30 KD was similar to the findings of Dharmakrong and Sirisinha (1983). Standardization of the quanity of the antibody used was not a practical possibility because of the scarcity of antigen.

Table 5.1 The approximate molecular weights of peptide bands bound to the respective antibodies by Western Blot.

	Antigens		
	T. cati	*T. canis*	*A. cantonensis*
Antibodies			
T. cati		105,818	30,390
		101,120	
		92,368	
T. canis		228,644	
		106,980	
		99,628	30,390
		67,745	
		32,652	
		28,998	
A. cantonensis	-	-	-
Control	-	-	-

5.4 THE ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

5.4.1 Introduction

The Elisa has proved a sensitive and simple assay for serum antibody. The important development was the linkage of soluble antigen (or antibody) to a solid phase while retaining the reactivity of the immunological component (Engvall and Perlman 1971; Engvall 1980). Voller (1976) first used a plastic tube to absorb the antigen for Elisa test for malaria but found that soft vinyl was easier for large scale use (Fig. 5.11). Kelsoe and Weller (1978) and Schinski (1976) carried out very elegant controlled studies comparing Elisa with other tests and found that the Elisa was as sensitive as radioimmunoassay and was more sensitive than both indirect fluorescent antibody tests and indirect haemagglutination assays (Voller *et al.* 1979). The wide immunological cross-reactivity between different helminths has complicated the application of serology to helminth diseases. A very elegant Elisa method has been developed for toxocariasis by de Savigny (1979) specific the secretory antigens from this helminth. The test is highly specific and can be used to indicate infection or past experience of man with toxocariasis (van Knapen, van Leusalen, Polderman 1983; Nicholas, Stewart and Walker 1986).

5.4.2 Materials and Methods

Absorption of Antigen to the Solid Phase

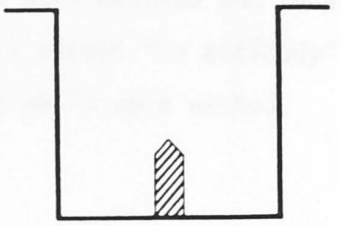
The optimum concentrations of antigens used in Elisa are normally in the range of 1 µg to 10 µg/ml protein. Polyvinyl microtitre plates type 220-9 (Dynatech Laboratories, Alexandria USA) 12.7 x 9.5 cm were coated with antigen diluted in 0.04 M phosphate buffer at pH 7.4 overnight at 4°C. (All 96 wells except "no antigen" control were filled with antigen).

Fig. 5.11

ELISA

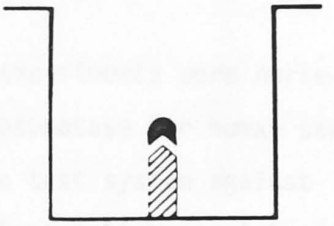
The Indirect Method For Assay Of Antibody

1 Antigen adsorbed to plate



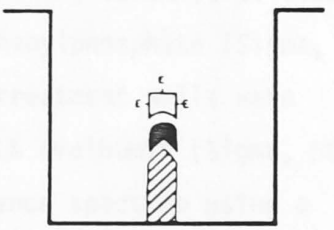
wash

2 Add serum : any specific antibody attaches to antigen



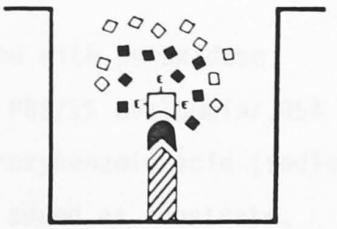
wash

3 Add enzyme labelled antiglobulin which attaches to antibody



wash

4 Add substrate



Amount hydrolysed ≡ amount antibody present

(after Voller et al 1979)

Addition of Serum Samples

The time and temperature at which samples are incubated are important. As with antigen, the optimum dilution was determined by preliminary titration. The antigen coated wells were emptied and 25 µl PBS/Tween 20 diluted serum was added to all wells except "no antibody" controls and incubated at 37°C for an hour. The wells were washed using (PBS/Tween 20) solution.

Addition of Enzyme Linked Antiglobin Conjugate and Enzyme Specific Substrate

The enzyme most commonly used in these experiments were horseradish peroxidase for mouse sera and alkaline phosphatase for human sera. To detemine optimum dilution for the conjugate, a test system against reference positive and negative sera was used. Goat anti-human IgG conjugated with alkaline phosphatase (Mils-Yeda, Rehovot, Israel), diluted 1:500 with PBS, 2 hr at 20°C, disodium p-nitropehenylphosphate (Sigma, St Louis, MD USA) 20 min at 20°C. Between each treatment wells were washed repeatedly with PBS plus 0.05% Tween 20 .1% ovalbumin (Sigma, St Louis, MD USA). Absorbance was read as a difference spectrum using a Titertek Multiskan spectrophotometer at 405 and 620 nm.

Similarly, goat anti-mouse IgG conjugated with peroxidase (Miles-Yeda, Rehovot, Israel) diluted 1:500 with PBS/1% ovalbumin/.05% Tween 20, 2 hr at 20°C, 4-amino antipyrine P-hydroxybenzoic acid (sodium salt) (Sigma, St Louis USA) and 0.0015% H_2O_2 was added as substrate. The absorbance was read as a difference spectrum using the Titertek at 510 and 620 nm.

5.4.3 Results

Three sets of polyvinyl plates were coated with *T. canis*, *T. cati*, *T. pteropodis* and *Angiostrongylus catonensis* antigens and incubated with human and mice sera. The cross reactivity and the

relationship between them (Figs. 5.12-16). Fig. 5.12 is a typical titration curve for positive and negative *T. canis* sera. (Fig. 5.13 compares a *T. canis* positive titration curve with a cross reacting *T. pteropodis* antibody, whereas *T. pteropodis* antigen/antibody complexes gave a negative photometric absorbance. Fig. 5.14 show the binding ability of *T. pteropodis* antigen to human IgG.

5.4.4 Discussion

Clinically positive *T. canis* human and mouse sera had shown high titre values (Fig. 5.14). *T. canis, T. cati* and *T. pteropodis* have cross reacted with each other which is consistent with previous findings of Nicholas, Stewart and Mitchell (1984) (Fig. 5.13). However, higher dilutions of sera shows that the cross reaction does not invalidate the immunodiagnosis (Fig. 5.15). The quantity of protein stimulated was quite high in *T. pteropodis* compared to other antigens. The *T. pteropodis* seemed to show a weak binding. This was observed in the previous experiments. *T. pteropodis* antigen and *T. pteropodis* +ve antibody have individually proved their binding capacity with *T. canis* +ve antibody and *T. canis* antigen respectively (Fig. 5.13, 5.14). *T. pteropodis* and *T. pteropodis* +ve antibody failed to give positive titre values and may be due to variation in binding. This demands more detail study of *T. pteropodis* antigen antibody complexes. Fig. 5.16 shows that there was a positive cross reaction between *T. canis* antisera and *A. cantonensis* antigen. Human antisera to *T. canis* and *A. cantonensis* gave negative Elisa to *A. cantonensis* ES antigen. This could be due to degrading *A. cantonensis* antigen.

Fig. 5.12 A typical titration curve for *T. canis* positive and negative sera tested by indirect Elisa

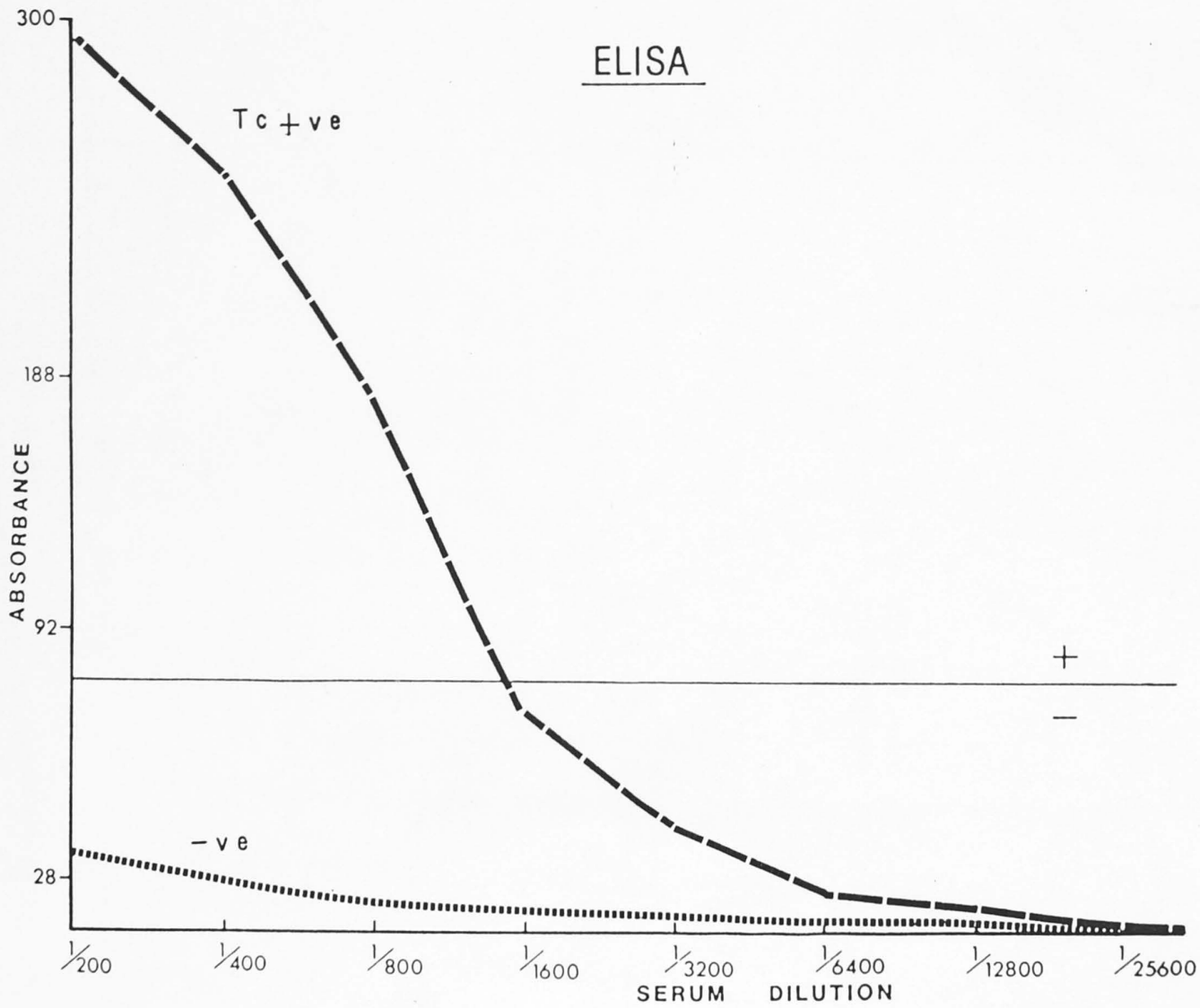

ELISA
300
188
92
28
ABSORBANCE
Tc +ve
-ve
+
-
1/200
1/400
1/800
1/1600
1/3200
1/6400
1/12800
1/25600
SERUM DILUTION

Fig. 5.13 Elisa showing the cross reaction between *T. canis* and *T. pteropodis*

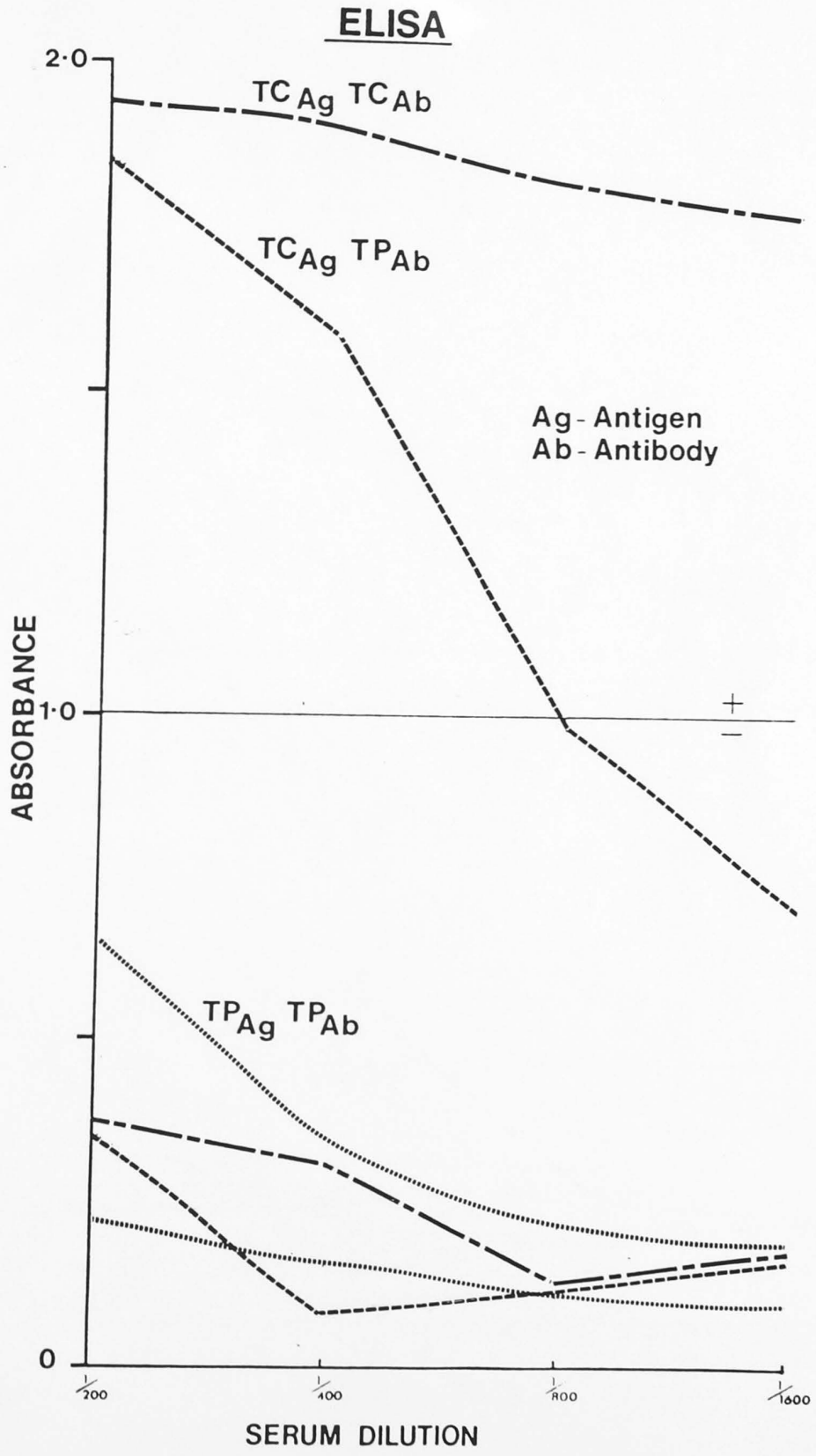

ELISA
2·0
1·0
0
ABSORBANCE
TC Ag TC Ab
TC Ag TP Ab
TP Ag TP Ab
Ag - Antigen
Ab - Antibody
+
−
1/200
1/400
1/800
1/1600
SERUM DILUTION

Fig. 5.14 An Elisa showing *T. canis* and *T. pteropodis* binding to *T. canis* positive human sera

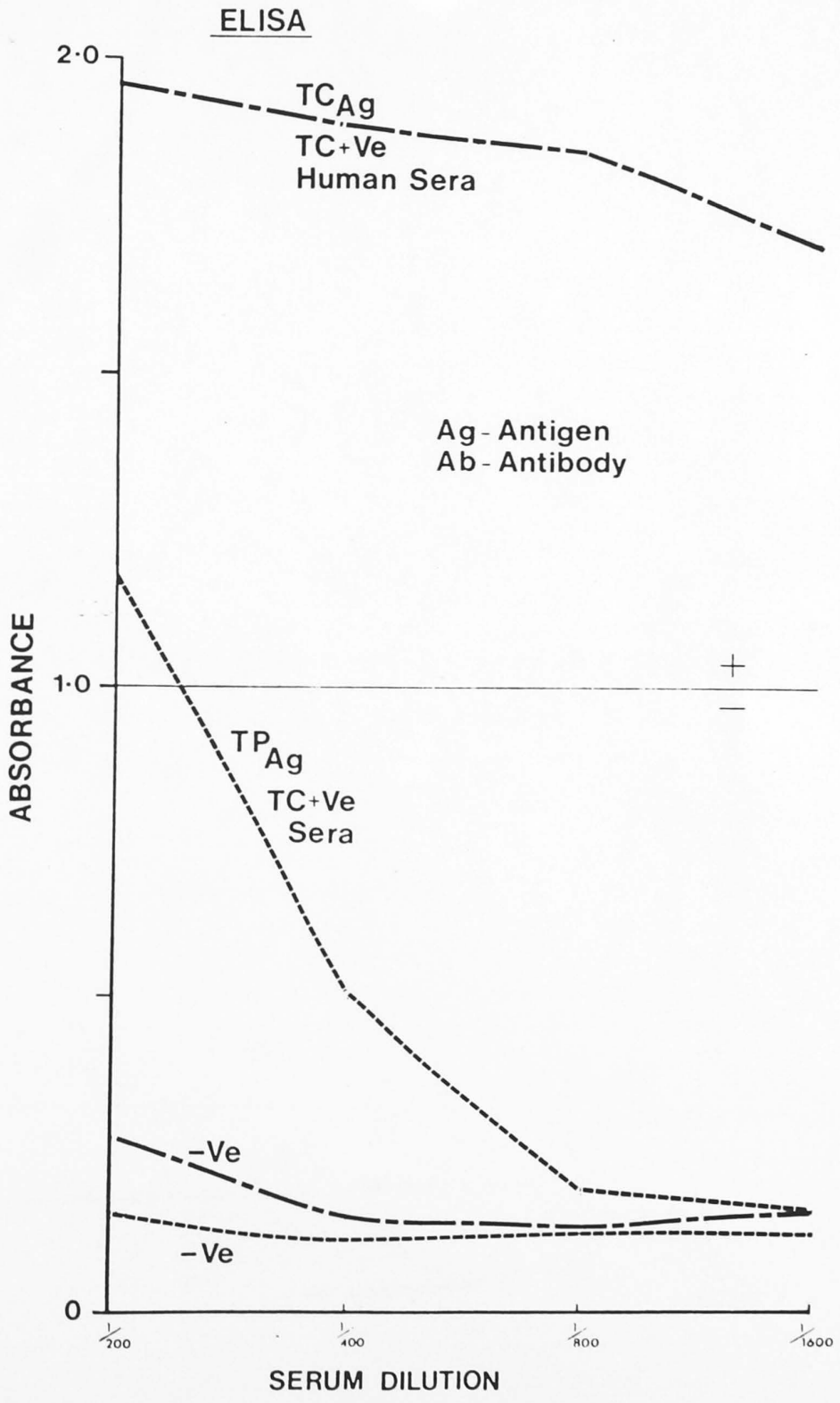

ELISA
2·0
TC Ag
TC+Ve
Human Sera
Ag - Antigen
Ab - Antibody
ABSORBANCE
1·0
+
—
TP Ag
TC+Ve
Sera
-Ve
-Ve
0
1/200
1/400
1/800
1/1600
SERUM DILUTION

Fig. 5.15 An Elisa absorbance plot showing the degree of cross reaction between *T. canis* and *T. cati*

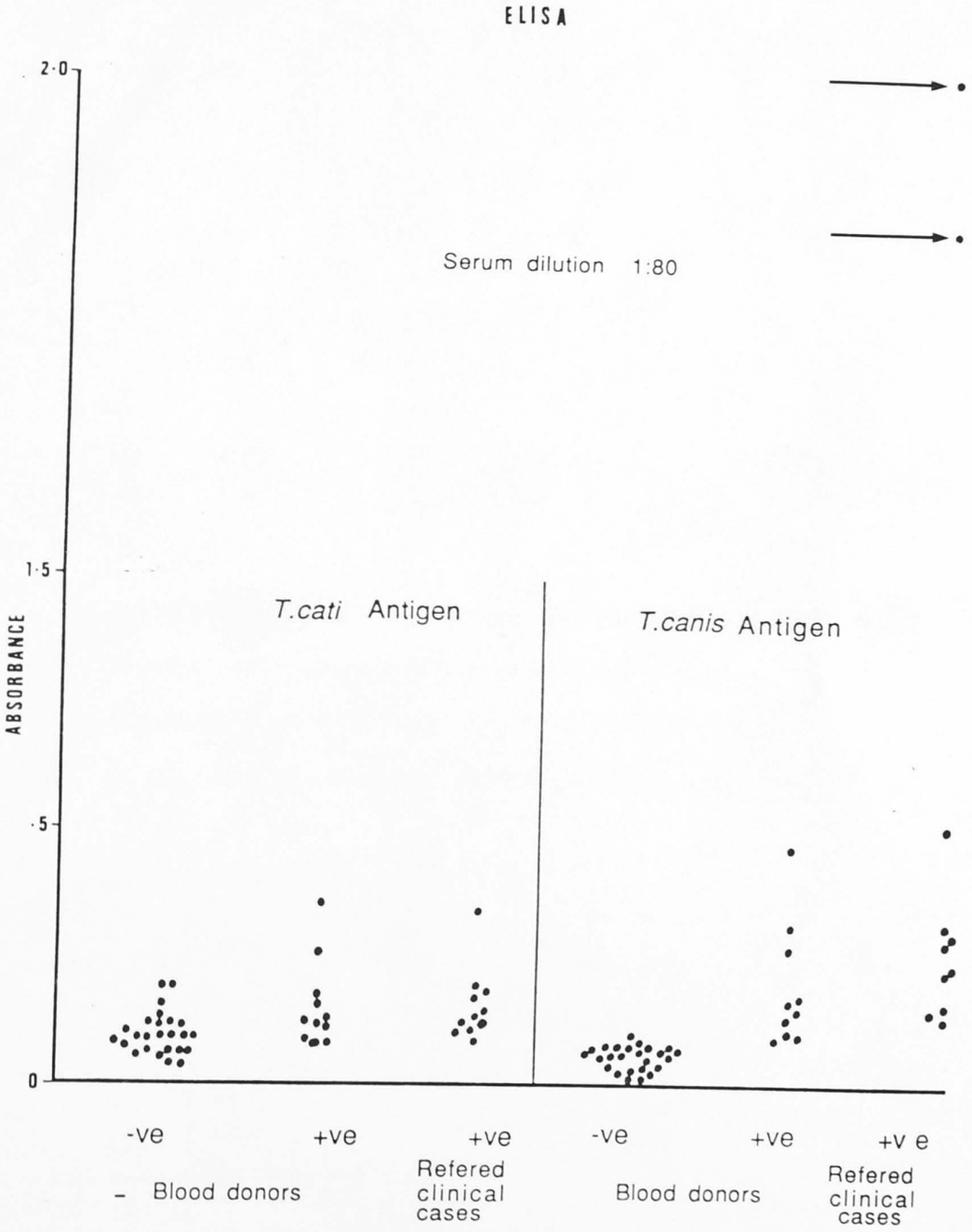
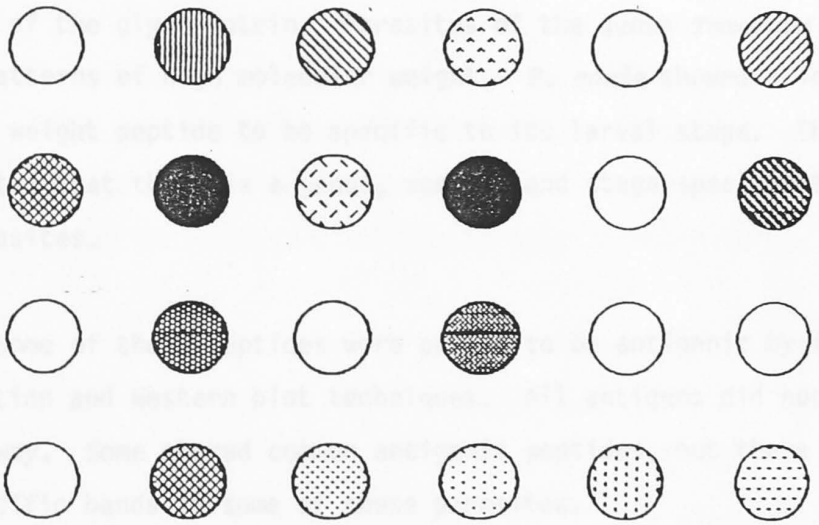

ELISA
2·0
1·5
·5
0
ABSORBANCE
Serum dilution 1:80
T.cati Antigen
T.canis Antigen
-ve
+ve
+ve
-ve
+ve
+v e
Blood donors
Refered clinical cases
Blood donors
Refered clinical cases

Fig. 5.16 Schematic representation of Elisa absorbance showing the cross reaction between *T. canis*, *T. cati*, *A. cantonensis* and *D. immitis* when titrated with human positive for *T. canis* and *A. cantonensis* sera

ELISA TEST



- ve	+ ve	+ ve	+ ve	control	+ve
	T.canis	A.cant.	T.canis		T.canis

Clinically positive Human sera

.112	.043	.093	.086	.076	.052	.085
.086	.057	>2	.082	>2	.044	.267
.042	.017	.184	.006	.143	.012	.028
.089	.047	.096	.09	.095	.063	.062

mean +ve 0.1741
mean -ve 0.01726

5.5 CONCLUSIONS

It is confirmed that nematodes excrete or secrete proteins into the media from the cuticle as well as their natural orifices. From previous studies they would appear to be glycoproteins. This thesis analyses their constituent peptides and antigenicity by different methods. Nematodes are found to share similar patterns of molecular weight peptides. This reflects the probability of similar glycoproteins in their extracts. Biochemically the antigen reacts depending on the structure of the glycoprotein. Parasites of the genus *Toxocara* showed peptide patterns of high molecular weight. *T. canis* showed a low molecular weight peptide to be specific to its larval stage. This demonstrates that there is a genus, species and stage specificity among these parasites.

Some of these peptides were proved to be antigenic by immunoprecipitation and Western blot techniques. All antigens did not react the same way. Some shared common antigenic peptides, but there were other specific bands in some of these parasites.

Antigens extracted from *Toxocara* worms were found to be stable when compared with filarial and *Angiostrongylus* parasites. Filarial worms were collected in the presence of blood and furthermore release of cathepsin P like enzyme from the filarial cuticle could have been the cause of proleolytic breakdown. It has been found that more antigens can be obtained by *in vivo* culture of the *T. canis* larva in a rabbit and harvesting the larvae from lungs. The *in vitro* culture could be maintained for longer periods by incorporating a mammalian growth hormone factor (De Savigny, pers. comm.).

The Elisa tests carried out in our laboratory have shown that the cross reactions can be overcome by optimum dilution. Antigens can

be purified by immunoaffinity chromatography. A specific antigen can be isolated by Western blot. Isolation of an appropriate epitope is essential for immunodiagnosis.

In all the experiments quantifying protein does not measure the antigenic component alone. The protein component from glycoprotein could be separated by periodate treatment which will enable a better standardisation procedure.

The function of those antigens are not clear and there is need for more studies in this field. The availability of the parasite antigen is an ongoing problem for parasitologists. This could be overcome by producing the necessary epitope by genetic engineering.

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Appendix I.

Standard Protein Markers (Sigma)

MW-SDS-200 - For molecular weights 30,000 to 200,000

Protein	Approximate Molecular Weight
Carbonic anhydrase	29,000
Albumin, egg	45,000
Albumin, bovine	66,000
Phosphorylase B	97,400
β-galactosidase	116,000
Myosin	205,000

MW-SDS-701 - For molecular weights 14,000 to 70,000

α-lactalbumin	14,200
Trypsin inhibitor	20,100
Trypsinogen	24,000
Carbonic anhydrase	29,000
Glyceraldehyde-3-phosphate	36,000
Albumin, egg	45,000
Albumin, bovine	66,000

Appendix II.

Relative mobility (RF) and molecular weights of polypeptides obtained from different parasites.

Gel No.

1. *T. canis* adult ES (CB)*
2. *T. canis* adult (cuticular extract using DOC)* (CB)
3. *T. canis* adult (DOC) (^{125}I)
4. *T. canis* adult (DOC) (CB)
5. *T. canis* adult ES (^{125}I)
6. *T. canis* adult ES (^{125}I)
7. *T. canis* adult ES (^{125}I)
8. *T. canis* adult PSMF (CB)
9. *T. canis* ligatured (^{125}I)
10. *T. canis* larval (L_2) (CB)
11. *T. canis* ES (CB)
12. *T. canis* (L_2) (^{35}S)
13. *T. canis* (L_2) sonicated
14. *T. canis* adult (CB)
15. *T. canis* L_2 (DOC) (CB)
16. *T. canis* L (DOC) (CB)
17. *T. canis* L ES (^{125}I)
18. *T. canis* L_2 sonicated (CB)
19. *T. leonina* adult ES (CB)
20. *T. cati* adult ES (^{125}I)
21. *T. cati* L_2 ES (^{35}S)
22. *T. cati* adult (DOC) (^{125}I)
23. *T. pteropodis* (L) ES (^{35}S)
24. *T. pteropodis* (L) ES (^{35}S)
25. *T. pteropodis* (L) ES (^{35}S)
26. *A. suum* ES (^{125}I)

Gel No.

27. *A. suum* (DOC) (^{125}I)
28. *A. suum* (L) ES (^{35}S)
29. *A. suum* (L) sonicated (^{35}S)
30. *A. suum* (L) sonicated (^{35}S)
31. *D. immitis* ES (CB)
32. *D. immitis* (DOC) (CB)
33. *D. immitis* ES (^{125}I)
34. *D. immitis* sonicated (CB)
35. *D. immitis* microfilaria (DOC) (^{35}S)
36. *D. immitis* somatic (^{125}I)
37. *D. immitis* (DOC) (CB)
38. *D. immitis* somatic (CB)
39. *D. roemeri* (DOC) (^{125}I)
40. *D. roemeri* ES (^{125}I)
41. *D. roemeri* (DOC) (CB)
42. *D. roemeri* ES (CB)
43. *A. cantonensis* L_3 (^{35}S) (20°C)
44. *A. cantonensis* L_3 (^{35}S)
45. *A. cantonensis* L_3 (^{35}S)
46. *A. cantonensis* (L_3 (^{35}S) (37°C)
47. *A. cantonensis* (L_3
48. *A. cantonensis* adult (DOC) (^{125}I)
49. *A. cantonensis* L_3 (^{35}S)
50. *A. cantonensis* L_3

*CB = Coomassie Blue

*DOC = deoxycolate

Appendix IIA.

RF values and molecular weights of different polypeptides obtained from different parasites corresponding to the respective gels in Appendix II. Molecular weights predicted by $Y = 1.048\ x + 5.3616$, $R^2 = .9112$.

1

Rf	Log MW	Molecular Wt
0.52	4.817	65560
0.54	4.796	62471
0.55	4.785	60982
0.57	4.764	58109
0.58	4.754	56723
0.69	4.638	43499
0.75	4.576	37636
0.80	4.523	33358
0.83	4.492	31028
0.85	4.471	29566

2

0.35	4.995	98810
0.46	4.880	75774
0.51	4.827	67161
0.55	4.785	60982
0.65	4.680	47907
0.69	4.638	43499
0.73	4.597	39497
0.75	4.576	37636
0.96	4.356	22674

3

0.28	5.068	116993
0.36	4.984	96454
0.44	4.900	79521
0.56	4.775	59528
0.76	4.565	36738

4

0.13	5.225	168020
0.14	5.215	164014
0.21	5.142	138522
0.24	5.110	128849
0.28	5.068	116993
0.35	4.995	98810
0.41	4.932	85491
0.53	4.806	63997
0.70	4.628	42462

5

0.63	4.701	50276
0.66	4.670	46765
0.88	4.439	27502
0.92	4.397	24971
0.95	4.366	23227
0.96	4.356	22674

6

0.15	5.204	160103
0.28	5.068	116993
0.32	5.026	106228
0.39	4.953	89718
0.49	4.848	70482
0.56	4.775	59528
0.64	4.691	49077
0.68	4.649	44562
0.71	4.618	41450

7

0.18	5.173	148922
0.32	5.026	106228
0.50	4.838	68802
0.57	4.764	58109
0.68	4.649	44562
0.73	4.597	39497
0.84	4.481	30289

8

0.27	5.079	119851
0.46	4.880	75774
0.57	4.764	58109
0.64	4.691	49077
0.75	4.576	37636
0.80	4.523	33358
0.86	4.460	28862
0.99	4.324	21090

9

0.09	5.267	185046
0.11	5.246	176327
0.21	5.142	138522
0.28	5.068	116993
0.32	5.026	106228
0.35	4.995	98810
0.36	4.984	96454
0.39	4.953	89718
0.43	4.911	81463
0.50	4.838	68802
0.53	4.806	63997
0.61	4.722	52762
0.70	4.628	42462
0.78	4.544	35007

10

0.25	5.100	125777
0.30	5.047	111481
0.32	5.026	106228
0.60	4.733	54051
0.85	4.471	29566

11

0.38	4.963	91909
0.42	4.921	83453
0.50	4.838	68802
0.62	4.712	51504
0.77	4.555	35862

12

0.33	5.016	103696
0.34	5.005	101223
0.38	4.963	91909
0.40	4.942	87579
0.51	4.827	67161
0.65	4.680	47907
0.86	4.460	28862
0.89	4.429	26846
0.94	4.376	23795

13

0.11	5.246	176327
0.29	5.058	114204
0.30	5.047	111481
0.33	5.016	103696
0.34	5.005	101223
0.38	4.963	91909
0.51	4.827	67161
0.55	4.785	60982
0.62	4.712	51504
0.64	4.691	49077
0.70	4.628	42462
0.74	4.586	38555
0.80	4.523	33358
0.86	4.460	28862
0.89	4.429	26846
0.94	4.376	23795

14

0.11	5.246	176327
0.34	5.005	101223
0.37	4.974	94154
0.40	4.942	87579
0.43	4.911	81463
0.47	4.869	73967
0.50	4.838	68802
0.67	4.659	45650
0.70	4.628	42462
0.71	4.618	41450
0.77	4.555	35862
0.80	4.523	33358
0.83	4.492	31028
0.84	4.481	30289
0.90	4.418	26206

15

0.35	4.995	98810
0.46	4.880	75774
0.51	4.827	67161
0.55	4.785	60982
0.63	4.701	50276
0.73	4.597	39497
0.92	4.397	24971
0.96	4.356	22674

16

0.36	4.984	96454
0.38	4.963	91909
0.42	4.921	83453
0.44	4.900	79521
0.82	4.502	31786
0.93	4.387	24376

17

0.06	5.299	198939
0.13	5.225	168020
0.24	5.110	128849
0.28	5.068	116993
0.35	4.995	98810
0.36	4.984	96454
0.45	4.890	77625
0.53	4.806	63997
0.70	4.628	42462
0.75	4.576	37636

18

0.12	5.236	172123
0.35	4.995	98810
0.42	4.921	83453
0.46	4.880	75774
0.48	4.859	72204
0.51	4.827	67161
0.55	4.785	60982
0.55	4.785	60982
0.58	4.754	56723
0.63	4.701	50276
0.69	4.638	43499
0.73	4.597	39497
0.80	4.523	33358
0.85	4.471	29566
0.88	4.439	27502
0.92	4.397	24971
0.96	4.356	22674

19

0.05	5.309	203798
0.09	5.267	185046
0.21	5.142	138522
0.24	5.110	128849
0.26	5.089	122778
0.32	5.026	106228
0.40	4.942	87579
0.47	4.869	73967
0.52	4.817	65560
0.57	4.764	58109
0.62	4.712	51504
0.74	4.586	38555
0.80	4.523	33358
0.90	4.418	26206

20

0.04	5.320	208776
0.30	5.047	111481
0.35	4.995	98810
0.37	4.974	94154
0.39	4.953	89718
0.40	4.942	87579
0.57	4.764	58109
0.70	4.628	42462
0.87	4.450	28173
0.92	4.397	24971
0.98	4.335	21605

21

0.06	5.299	198939
0.09	5.267	185046
0.45	4.890	77625
0.53	4.806	63997
0.56	4.775	59528
0.70	4.628	42462

22

0.01	5.351	224450
0.03	5.330	213875
0.36	4.984	96454
0.44	4.900	79521
0.80	4.523	33358
0.91	4.408	25581
0.93	4.387	24376

23

0.02	5.341	219099
0.06	5.299	198939
0.34	5.005	101223
0.36	4.984	96454
0.50	4.838	68802
0.58	4.754	56723
0.62	4.712	51504
0.76	4.565	36738
0.85	4.471	29566
0.93	4.387	24376

24

0.30	5.047	111481
0.33	5.016	103696
0.34	5.005	101223
0.55	4.785	60982
0.67	4.659	45650
0.70	4.628	42462
0.80	4.523	33358
0.83	4.492	31028
0.86	4.460	28862
0.94	4.376	23795

25

0.08	5.278	189566
0.23	5.121	131996
0.27	5.079	119851
0.31	5.037	108823
0.36	4.984	96454
0.38	4.963	91909
0.46	4.880	75774
0.52	4.817	65560
0.54	4.796	62471
0.58	4.754	56723
0.59	4.743	55371
0.65	4.680	47907
0.69	4.638	43499
0.73	4.597	39497
0.80	4.523	33358
0.92	4.397	24971
0.97	4.345	22133

26

0.03	5.330	213875
0.14	5.215	164014
0.21	5.142	138522
0.23	5.121	131996
0.25	5.100	125777
0.30	5.047	111481
0.33	5.016	103696
0.45	4.890	77625
0.51	4.827	67161
0.62	4.712	51504
0.69	4.638	43499
0.88	4.439	27502

27

0.03	5.330	213875
0.25	5.100	125777
0.62	4.712	51504
0.69	4.638	43499
0.88	4.439	27502

28

0.03	5.330	213875
0.42	4.921	83453
0.46	4.880	75774
0.50	4.838	68802
0.54	4.796	62471
0.79	4.534	34173
0.88	4.439	27502
0.98	4.335	21605

29

0.38	4.963	91909
0.40	4.942	87579
0.51	4.827	67161
0.55	4.785	60982
0.74	4.586	38555
0.80	4.523	33358

30

0.13	5.225	168020
0.46	4.880	75774
0.48	4.859	72204
0.56	4.775	59528
0.79	4.534	34173

31

0.09	5.267	185046
0.21	5.142	138522
0.29	5.058	114204
0.32	5.026	106228
0.38	4.963	91909
0.41	4.932	85491
0.45	4.890	77625
0.52	4.817	65560
0.57	4.764	58109
0.64	4.691	49077
0.66	4.670	46765
0.95	4.366	23227
0.96	4.356	22674
0.99	4.324	21090

32

0.07	5.288	194196
0.14	5.215	164014
0.26	5.089	122778
0.37	4.974	94154
0.47	4.869	73967
0.51	4.827	67161
0.59	4.743	55371
0.64	4.691	49077
0.71	4.618	41450
0.85	4.471	29566
0.99	4.324	21090

33

0.11	5.246	176327
0.32	5.026	106228
0.71	4.618	41450

34

0.09	5.267	185046
0.14	5.215	164014
0.18	5.173	148922
0.25	5.100	125777
0.27	5.079	119851
0.28	5.068	116993
0.34	5.005	101223
0.38	4.963	91909
0.40	4.942	87579
0.42	4.921	83453
0.43	4.911	81463
0.47	4.869	73967
0.49	4.848	70482
0.54	4.796	62471
0.57	4.764	58109
0.59	4.743	55371
0.64	4.691	49077
0.68	4.649	44562
0.71	4.618	41450
0.75	4.576	37636
0.81	4.513	32563
0.84	4.481	30289
0.88	4.439	27502
0.92	4.397	24971
0.96	4.356	22674
0.98	4.335	21605

35

0.11	5.246	176327
0.24	5.110	128849
0.28	5.068	116993
0.32	5.026	106228
0.39	4.953	89718
0.49	4.848	70482
0.57	4.764	58109
0.60	4.733	54051
0.64	4.691	49077
0.68	4.649	44562
0.86	4.460	28862

36

0.09	5.267	185046
0.13	5.225	168020
0.14	5.215	164014
0.21	5.142	138522
0.35	4.995	98810
0.36	4.984	96454
0.41	4.932	85491
0.45	4.890	77625
0.53	4.806	63997
0.59	4.743	55371
0.70	4.628	42462
0.75	4.576	37636
0.83	4.492	31028

37

0.47	4.869	73967
0.48	4.859	72204
0.52	4.817	65560
0.54	4.796	62471
0.58	4.754	56723
0.62	4.712	51504
0.64	4.691	49077
0.69	4.638	43499
0.71	4.618	41450
0.75	4.576	37636
0.77	4.555	35862
0.82	4.502	31786
0.94	4.376	23795
0.98	4.335	21605

38

0.25	5.100	125777
0.30	5.047	111481
0.37	4.974	94154
0.42	4.921	83453
0.44	4.900	79521
0.52	4.817	65560
0.59	4.743	55371
0.64	4.691	49077
0.67	4.659	45650
0.70	4.628	42462
0.74	4.586	38555
0.81	4.513	32563
0.89	4.429	26846
0.90	4.418	26206
0.95	4.366	23227
0.96	4.356	22674

39

0.21	5.142	138522
0.28	5.068	116993
0.29	5.058	114204
0.39	4.953	89718
0.49	4.848	70482
0.56	4.775	59528
0.71	4.618	41450
0.84	4.481	30289

40

0.28	5.068	116993

41

0.34	5.005	101223
0.36	4.984	96454
0.48	4.859	72204
0.53	4.806	63997
0.57	4.764	58109
0.62	4.712	51504
0.72	4.607	40461
0.79	4.534	34173
0.92	4.397	24971
0.97	4.345	22133

42

0.35	4.995	98810
0.37	4.974	94154
0.48	4.859	72204
0.53	4.806	63997

43

0.05	5.309	203798
0.11	5.246	176327
0.17	5.183	152560
0.22	5.131	135220
0.27	5.079	119851
0.30	5.047	111481
0.34	5.005	101223
0.36	4.984	96454
0.37	4.974	94154
0.45	4.890	77625
0.49	4.848	70482
0.51	4.827	67161
0.52	4.817	65560
0.56	4.775	59528
0.58	4.754	56723
0.60	4.733	54051
0.64	4.691	49077
0.68	4.649	44562
0.73	4.597	39497
0.77	4.555	35862
0.82	4.502	31786
0.88	4.439	27502
0.91	4.408	25581
0.95	4.366	23227

44

0.30	5.047	111481
0.47	4.869	73967
0.62	4.712	51504
0.74	4.586	38555
0.84	4.481	30289
0.94	4.376	23795

45

0.38	4.963	91909
0.47	4.869	73967
0.51	4.827	67161
0.57	4.764	58109
0.61	4.722	52762
0.64	4.691	49077
0.70	4.628	42462
0.74	4.586	38555
0.76	4.565	36738
0.80	4.523	33358
0.92	4.397	24971
0.96	4.356	22674

46

0.17	5.183	152560
0.22	5.131	135220
0.27	5.079	119851
0.30	5.047	111481
0.34	5.005	101223
0.36	4.984	96454
0.37	4.974	94154
0.45	4.890	77625
0.49	4.848	70482
0.54	4.796	62471
0.58	4.754	56723
0.64	4.691	49077
0.68	4.649	44562
0.73	4.597	39497
0.77	4.555	35862
0.82	4.502	31786
0.88	4.439	27502
0.91	4.408	25581
0.95	4.366	23227

47

0.07	5.288	194196
0.33	5.016	103696
0.47	4.869	73967
0.62	4.712	51504
0.66	4.670	46765
0.77	4.555	35862
0.84	4.481	30289
0.95	4.366	23227

48

0.08	5.278	189566
0.11	5.246	176327
0.15	5.204	160103
0.28	5.068	116993
0.32	5.026	106228
0.36	4.984	96454
0.44	4.900	79521
0.56	4.775	59528
0.60	4.733	54051
0.66	4.670	46765
0.72	4.607	40461
0.79	4.534	34173
0.90	4.418	26206

49

0.27	5.079	119851
0.28	5.068	116993
0.32	5.026	106228
0.36	4.984	96454
0.40	4.942	87579
0.42	4.921	83453
0.43	4.911	81463
0.52	4.817	65560
0.53	4.806	63997
0.54	4.796	62471
0.55	4.785	60982
0.56	4.775	59528
0.62	4.712	51504
0.77	4.555	35862
0.87	4.450	28173

50

0.18	5.173	148922
0.20	5.152	141906
0.24	5.110	128849
0.28	5.068	116993
0.32	5.026	106228
0.34	5.005	101223
0.37	4.974	94154
0.39	4.953	89718
0.42	4.921	83453
0.43	4.911	81463
0.46	4.880	75774
0.48	4.859	72204
0.51	4.827	67161
0.54	4.796	62471
0.59	4.743	55371
0.65	4.680	47907
0.68	4.649	44562
0.71	4.618	41450
0.74	4.586	38555
0.75	4.576	37636
0.79	4.534	34173
0.84	4.481	30289
0.87	4.450	28173
0.89	4.429	26846
0.92	4.397	24971

Appendix III. Variation within gels.

X_1 : Ag 1/Gel 1

Mean:	Std. Dev.:	Std. Error:	Variance:	Coef. Var.:	Count:
.105	.023	.01	5.502E-4	22.287	6
Minimum:	Maximum:	Range:	Sum:	Sum Squared:	# Missing:
.08	.142	.062	.632	.069	0
t 95%:	95% Lower:	95% Upper:			
.025	.081	.13			

1

X_2 : Ag 1/Gel 2

Mean:	Std. Dev.:	Std. Error:	Variance:	Coef. Var.:	Count:
.235	.042	.017	1.793E-3	18.057	6
Minimum:	Maximum:	Range:	Sum:	Sum Squared:	# Missing:
.192	.292	.1	1.407	.339	0
t 95%:	95% Lower:	95% Upper:			
.044	.19	.279			

2

X_3 : Ag 2/Gel 1

Mean:	Std. Dev.:	Std. Error:	Variance:	Coef. Var.:	Count:
.306	.05	.021	2.531E-3	16.434	6
Minimum:	Maximum:	Range:	Sum:	Sum Squared:	# Missing:
.256	.367	.111	1.837	.575	0
t 95%:	95% Lower:	95% Upper:			
.053	.253	.359			

3

X_4 : Ag 2/Gel 2

Mean:	Std. Dev.:	Std. Error:	Variance:	Coef. Var.:	Count:
.43	.059	.024	3.437E-3	13.646	6
Minimum:	Maximum:	Range:	Sum:	Sum Squared:	# Missing:
.364	.496	.132	2.578	1.125	0
t 95%:	95% Lower:	95% Upper:			
.062	.368	.491			

4

Appendix III (contd.)

X_5: Ag 3/Gel 1

Mean:	Std. Dev.:	Std. Error:	Variance:	Coef. Var.:	Count:
.626	.108	.044	.012	17.301	6
Minimum:	Maximum:	Range:	Sum:	Sum Squared:	# Missing:
.52	.747	.227	3.758	2.412	0
t 95%:	95% Lower:	95% Upper:			5
.114	.513	.74			

X_6: Ag 3/Gel 2

Mean:	Std. Dev.:	Std. Error:	Variance:	Coef. Var.:	Count:
.791	.149	.061	.022	18.771	6
Minimum:	Maximum:	Range:	Sum:	Sum Squared:	# Missing:
.608	.92	.312	4.749	3.869	0
t 95%:	95% Lower:	95% Upper:			6
.156	.636	.947			

Appendix III (contd.)

X_1 : Myosin

Mean:	Std. Dev.:	Std. Error:	Variance:	Coef. Var.:	Count:	
.132	.029	5.756E-3	8.284E-4	21.834	25	
Minimum:	Maximum:	Range:	Sum:	Sum Squared:	# Missing:	1
.08	.182	.102	3.296	.454	125	

X_2 : β Galact

Mean:	Std. Dev.:	Std. Error:	Variance:	Coef. Var.:	Count:	
.274	.042	8.377E-3	1.754E-3	15.27	25	
Minimum:	Maximum:	Range:	Sum:	Sum Squared:	# Missing:	2
.192	.333	.141	6.857	1.923	125	

X_3 : Phosphoryl

Mean:	Std. Dev.:	Std. Error:	Variance:	Coef. Var.:	Count:	
.343	.044	8.718E-3	1.900E-3	12.698	25	
Minimum:	Maximum:	Range:	Sum:	Sum Squared:	# Missing:	3
.256	.4	.144	8.582	2.992	125	

X_4 : Bovine Alb

Mean:	Std. Dev.:	Std. Error:	Variance:	Coef. Var.:	Count:	
.462	.054	.011	2.921E-3	11.701	25	
Minimum:	Maximum:	Range:	Sum:	Sum Squared:	# Missing:	4
.363	.539	.176	11.547	5.403	125	

X_5 : Egg Alb

Mean:	Std. Dev.:	Std. Error:	Variance:	Coef. Var.:	Count:	
.673	.081	.016	6.496E-3	11.981	25	
Minimum:	Maximum:	Range:	Sum:	Sum Squared:	# Missing:	5
.52	.783	.263	16.817	11.469	125	

X_6 : C Anhyd

Mean:	Std. Dev.:	Std. Error:	Variance:	Coef. Var.:	Count:	
.854	.119	.024	.014	13.91	25	
Minimum:	Maximum:	Range:	Sum:	Sum Squared:	# Missing:	6
.608	.983	.375	21.347	18.567	125	